Biodiversity conservation in semi-natural grasslands – The significance of genetic and epigenetic variation

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IF YOU CAN DREAM IT, YOU CAN DO IT.

(*Walt Disney*)

Menyanthes trifoliata L.

THIS THESIS IS COMPOSED OF THE FOLLOWING MANUSCRIPTS, WHICH ARE LARGELY IDENTICAL WITH THE MANUSCRIPTS SUBMITTED FOR PUBLICATION:

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CHAPTER 4

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SUMMARY

In Europe, semi-natural grasslands are characterized by an outstanding species-richness and an enormous ecosystem diversity. Abandonment of traditional land use practices led to a biodiversity decline on an unprecedented scale during the last decades. Consequently, the Habitats Directive was initiated to protect semi-natural grasslands and some of their characteristic species. Besides ecosystem and species diversity, comprehensive biodiversity conservation should also address genetic diversity. However, plant genetic resource conservation is usually not included in biodiversity conservation strategies and thus, effective conservation of plant genetic diversity is still in its infancy. Plant genetic resources should be properly understood to ensure the development of applicable conservation methods. Hence, the knowledge of the species' taxonomy, origin, and evolution as well as its major drivers of genetic variation are of central importance. Therefore, the present study focused on potential explanatory variables for genetic variation in six common semi-natural grassland plant species. Furthermore, the impact and extent of rapid DNA methylation patterns was examined in contrast to comparatively slow alterations of the genetic code between two contrasting habitats.

Chapter 1 provides a brief overview about the importance of semi-natural grassland habitats and their species' (epi)genetic variation against the background of biodiversity decline and conservation.

In the following two chapters, amplified fragment length polymorphism (AFLP) analyses were applied to identify potential drivers of genetic variation. More specifically, the impact of habitat age, surrounding landscape structure, local habitat quality, and population size on genetic diversity and differentiation was tested.

In chapter 2, the genetic composition of three common and widely distributed calcareous grassland plant species, *Asperula cynanchica* L., *Campanula rotundifolia* L. s. str., and *Linum catharticum* L., was investigated. No crucial impact of habitat age, habitat quality, or population size was observed. However, the distance to the nearest settlement, the total area of calcareous grasslands, and their connectivity turned out as key drivers of genetic diversity. Genetic diversity, therefore, strongly depended on the surrounding landscape structure. Since landscape structure is indirectly shaped by land use,

our study supports the observation that genetic variation is strongly affected by grazing and thereby arising gene flow patterns.

Moreover, the genetic composition of *Angelica sylvestris* L., *Filipendula ulmaria* (L.) Maxim., and *Succisa pratensis* MOENCH populations in litter meadows was examined in chapter 3. Habitat age revealed no influence on genetic variation patterns again. The impact of landscape structure, habitat quality, and population size on genetic diversity depended on species affiliation. Distance to the nearest settlement, habitat size, the total area of wet meadows, and their connectivity shaped genetic diversity patterns of *A. sylv*estris and *F. ulmaria* populations. Local habitat quality affected, moreover, the genetic diversity of *F. ulmaria*, while genetic diversity of *S. pratensis* populations was driven only by population size. The history of origin, but also current mowing with agricultural machines, caused and still cause gene flow among litter meadow populations. Hence, all explanatory variables underlay anthropogenic land use patterns and thereby arising man-made gene flow.

Chapter 4 focused on potential differences in genetic and epigenetic variation patterns of *Trifolium pratense* L. between two contrasting semi-natural grassland habitats, calcareous grasslands and oat-grass meadows. An additional objective was to identify possible drivers of genetic and epigenetic variation. By conducting AFLP and MSAP (methylation-sensitive amplification polymorphism) analyses, low levels of genetic and epigenetic differentiation among populations and between habitat types were observed. Genetic variation was significantly isolated by habitat dissimilarity, while epigenetic variation was not. Habitat affiliation revealed no significant impact on genetic or epigenetic diversity. Furthermore, genetic diversity was not affected by environment, while epigenetic diversity levels correlated significantly with soil moisture and soil pH. Genetic and epigenetic variation were not interdependent and thus, shaped by different environmental conditions. On the one hand, genetic variation was influenced by habitat specific environmental conditions induced by land use related disturbance and gene flow patterns. On the other hand, epigenetic variation was driven by challenging environmental conditions decreasing under drought and high pH, with the latter potentially resulting in phosphorus limitation.

In chapter 5, all findings were recapitulated and placed in the context of *in situ* plant genetic resource conservation. Strengths and limitations of all analyses were highlighted against the background of genetic reserve identification, establishment, and maintenance. The chapter dealt, moreover, with perspectives for future scientific research. Thus, a multi-species approach on a larger spatial scale may exclude ecologically determined variation and allow genetic resource conservation above species level. Additionally, both genetic and epigenetic variation patterns should be integrated in the process of genetic reserve identification to add a new dimension of complexity to the diversity and evolutionary potential of natural populations. International exchange of knowledge may, moreover, ensure and facilitate sustainable genetic resource conservation.

CHAPTER 1

GENERAL INTRODUCTION

Centaurea jacea agg.

Biodiversity, the variety of life, covers the entire biological hierarchy from molecules to ecosystems including individuals, genotypes, populations, species, etc. and all their interactions (Sarkar & Margules, 2002). The Convention on Biological Diversity (CBD, 1992) classified three levels of diversity: (i) ecosystem diversity (communities of species and their environment), (ii) species diversity (species richness), and (iii) genetic diversity (variation in genotypes and genes) (Figure 1.1) (Ramanatha Rao & Hodgkin, 2002). Moreover, biodiversity stands for the availability of natural resources for plant and animal breeding or genetic and medical engineering (Haila & Kouki, 1994). Thus, it represents a key component of sustainable development in social and economic human systems (Ramanatha Rao & Hodgkin, 2002). Nevertheless, human appropriation of natural resources, spread of pathogenic, exotic, and domestic animals and plants as well as modifications of habitats and climate reveal major threats to biodiversity (Naeem et al., 2012). As a consequence, ecosystems are rapidly losing functional, taxonomic, phylogenetic, and genetic diversity all over the world (Naeem et al., 2012).

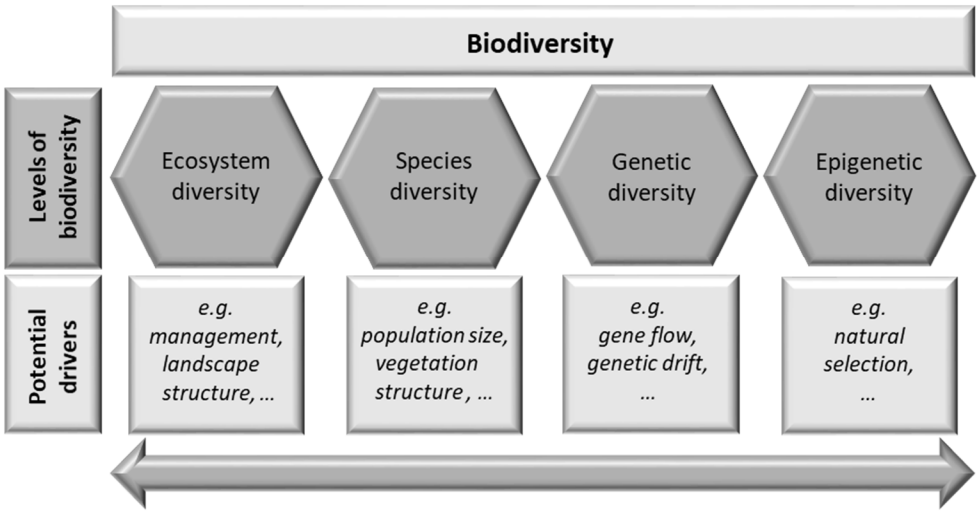


Figure 1.1: Levels of biodiversity and their potential drivers.

In Europe, semi-natural grasslands (Box 1.1) constitute the most diverse ecosystems hosting 18.1 % of Europe's endemic vascular plant species (Hobohm & Bruchmann, 2009) as well as a large proportion of the vertebrate and invertebrate fauna, e.g. two-thirds of the butterfly species (WallisDeVries & Van Swaay, 2009). Besides species-

richness, semi-natural grasslands provide a great amount of ecosystem services. Four main groups were defined by the Millennium Ecosystem Assessment (2005) and Hopkins (2009) highlighted the most important ones for semi-natural grasslands: (i) provisioning services: products (herbs, honey, dairy products, and meat), genetic material (seeds), and fresh water; (ii) supporting services: carbon fixation, soil formation, nutrient and water cycling; (iii) regulating services: stabilization of the natural environment by regulated air and water quality, soil erosion, and water run-off; and (iv) cultural services: aesthetic value and recreation areas. Therefore, semi-natural grasslands appear as key areas for biodiversity conservation in Europe (Raatikainen et al., 2009; Rosengren et al., 2013).

European semi-natural grasslands developed due to human activities (e.g. grazing, mowing, and burning) during the Anthropocene (Poschlod & WallisDeVries, 2002). Centuries of extensive, traditional land use led to an exceptional high species diversity (Figure 1.2) (Butaye et al., 2005; Poschlod & WallisDeVries, 2002). Nevertheless, semi-natural grasslands significantly decreased through traditional management abandonment during the last century (Poschlod et al., 2005). In 1998, Muller *et al.* (1998) named serious threats to semi-natural grasslands, which are still relevant. On the one hand, intensified grassland management with increased fertilizer application, early cutting, ploughing, drainage, or high grazing pressures homogenized species composition. On the other hand, abandonment led to a dominance of competitive species, eutrophication, and in many cases to subsequent reforestation. Besides management induced changes, semi-natural grasslands are destroyed by anthropogenic construction projects like highways, dams, or for leisure facilities. Atmospheric nitrogen deposition and invasive species constitute further threats (Habel et al., 2013).

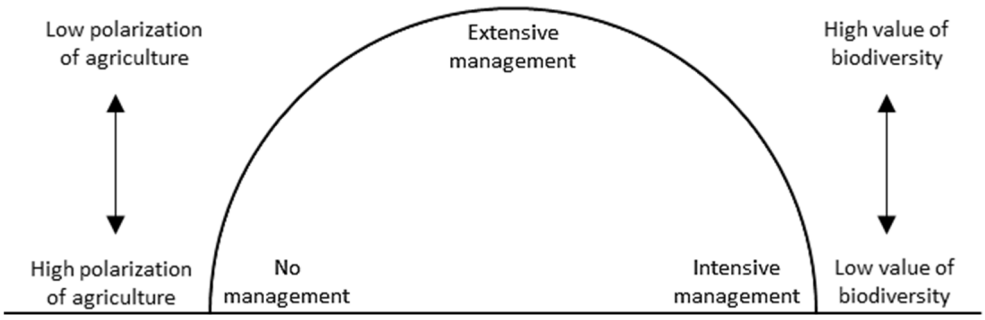


Figure 1.2: Relationship between polarization (towards intensification as well as abandonment) of agriculture and biodiversity values in semi-natural grasslands. Missing or intensified management decreases the conservation value regarding biodiversity. (Ostermann 1998; edited by Lehmair)

Semi-natural grasslands are subjected to similar threats by modern land use practices, although their ecosystems are characterized by different environmental settings (Box 1.1). These threats reduce habitat quality and quantity by pure habitat loss, individual patch size decline, and increasing fragmentation (Andrén, 1994; Poschlod & Schumacher, 1998). Consequently, even populations of common plant species become fewer, smaller, and more distant (Picó & Van Groenendael, 2007). They show reduced fitness levels due to several factors lowering genetic variation, such as limited pollen and seed dispersal, genetic drift, and inbreeding (Leimu et al., 2006; Luijten et al., 2000; Vergeer et al., 2003). In short term, small and fragmented populations become more susceptible to pathogens and herbivores (Brown, 1983; Ellstrand & Elam, 1993). In long term, their extinction risk increases especially among demographic stochasticity and unpredictable environmental conditions (Picó & Van Groenendael, 2007). Hence, fragmentation and downsizing of habitat patches jeopardise the persistence of highly diverse semi-natural grasslands (Fahrig, 2003; Raatikainen et al., 2009).

In response to these threats and to protect, inter alia, species-rich semi-natural grasslands, the European Commission passed the 'Council Directive 92/43/EEC on the conservation of natural habitats and of wild fauna and flora' (COM, 1992). A network of ecosystems with high conservation value, Natura 2000, was designed 'to halt the loss of biodiversity and the degradation of ecosystem services in the EU by 2020, and restore them as far as feasible, while stepping up the EU contribution to averting global biodiversity loss' (COM, 2011). Nevertheless, comprehensive biodiversity conservation should also address

genetic diversity (Ramanatha Rao & Hodgkin, 2002), which constitutes another key variable of biodiversity (Figure 1.1) (Laikre et al., 2010; May, 1994; Naeem et al., 2012; Wilkox, 1984).

On individual level, genetic diversity depicts genetic differences among individuals varying in DNA sequence, biochemical characteristics, physiological properties, and morphological characters (Ramanatha Rao & Hodgkin, 2002). On population level, it stands for the number of different alleles per population, their distribution, and their impact on populations' performance and distinctiveness (Ramanatha Rao & Hodgkin, 2002). Thus, genetic diversity represents the amount of genetic variation among individuals of populations, but also among populations of species (Brown, 1983). The variation that underpins genetic diversity is based on mutation processes, which are driven by recombination, genetic drift, gene flow, and natural selection (Ramanatha Rao & Hodgkin, 2002; Vellend, 2005).

When it comes to recombination, a species' mating system significantly shapes the genetic composition of its populations (Schmitt, 1983). Selfing species are often highly differentiated among populations with different alleles occurring in different populations (Tachida & Yoshimaru, 1996). Outcrossing species show a high degree of intrapopulation genetic structure due to regular recombination events (Baatout et al., 1990). The strong dependence on suitable mating partners and a greater susceptibility to small population size reveal a positive association between mean genetic diversity and fitness for outcrossing, but less for selfing species (Leimu et al., 2006; Picó & Van Groenendael, 2007).

Genetic drift represents random variation in gene frequencies caused by varying intensity and direction of selection, mutation, and gene exchange among populations (Dobzhansky & Pavlovsky, 1957; Wright, 1949). Random drift is generally not expected to contribute in directed evolutionary processes (Dobzhansky & Pavlovsky, 1957), but in small and isolated populations, genetic drift may reduce heterozygosity, change the populations' adaptive potential and their physiological optimum (Hooftman et al., 2003; Lande, 1976). Site connection by pollination and seed dispersal may rescue populations from genetic erosion and thus, counteract genetic drift (Brown & Kodric-Brown, 1977).

Gene flow provides new genetic material (new species or novel alleles at one or more loci) through pollen and seed dispersal (Vellend & Geber, 2005; Young et al., 1996). Both pollen and seeds may connect existing populations over great distances. Additionally, seeds enable the founding of new populations (Mix et al., 2006). Nevertheless, the exchange of pollen and seeds among populations greatly depends on dispersal vectors (Mix et al., 2006). Thus, plant-pollinator interactions may be limited by habitat fragmentation, since pollinating insects may rarely travel distances larger than 1 km (Kwak et al., 1998; Steffan-Dewenter & Tscharntke, 2002), while seed dispersal by animals may exceed distances of 100 km and more (Fischer et al., 1996; Manzano & Malo, 2006). However, gene flow among locally adapted populations could also provoke a short-term fitness decrease with non-local alleles increasing the migration load and thus, leading to outbreeding depression (Bradshaw, 1984).

Therefore, the interaction of gene flow and natural selection represents the adaptive potential of populations (McKay et al., 2005; Slatkin, 1985). Natural selection removes maladaptive alleles and increases mean population fitness by favouring locally adapted alleles (McKay et al., 2005). Thus, strong natural selection may overcome the effects of gene flow, which could limit or 'swamp' adaptive differentiation (McKay et al., 2005). Epigenetic variation (Figure 1.1), as a result of metastable DNA methylation, allows plant species to rapidly adapt and survive under challenging environmental conditions without changing their DNA sequence (Bossdorf et al., 2008; Herrera & Bazaga, 2011; Lira-Medeiros et al., 2010; Paun et al., 2010; Schulz et al., 2014, 2013; Wu et al., 2013). Epigenetic markers may, therefore, display the effects of natural selection (Hirsch et al., 2012) better than molecular markers, which are (nearly) neutral to natural selection (McKay et al., 2005).

Nowadays, semi-natural grassland populations are often spatially isolated and highly fragmented. Genetic habitat fragmentation by limited pollen and seed exchange restricts gene flow patterns (Honnay et al., 2006; Schmitt, 1983; Steffan-Dewenter & Tscharntke, 1999; Willerding & Poschlod, 2002) and increases, therefore, the likelihood of inbreeding depression, the accumulation of deleterious mutations, and the extent of genetic drift (Picó & Van Groenendael, 2007; Young et al., 1996). Consequently increased

genetic differentiation and reduced genetic diversity (Barrett & Kohn, 1991; McKay et al., 2005) may lower individual plant fitness and thus, increase their extinction risk (Ellstrand & Elam, 1993; Young et al., 1996). At worst, populations and even species may collapse due to genetic diversity loss (Frankham, 2005; Newman & Pilson, 1997). Hence, fragmented semi-natural grasslands suffer from species decline and changed community composition today (Butaye et al., 2005). Against the background of these threats, plant genetic resources should be properly understood, efficiently preserved, and carefully used (Ramanatha Rao & Hodgkin, 2002).

Nevertheless, plant genetic resources are usually not monitored (Laikre et al., 2010) and thus, most *in situ* or *ex situ* conservation efforts are conducted without sufficient information about genetic variation (Ramanatha Rao & Hodgkin, 2002). Keller *et al.* (2015) named several reasons for this gap in knowledge: comparable high costs of molecular analyses, limited understanding of how to implement findings about genetic composition in practical conservation strategies, and, above all, limited communication between scientists and practitioners. Thus, consistent national or regional biodiversity monitoring and the exchange of collected data are often constrained by inconsistent scientific methods and conservation aims (Pereira et al., 2013).

Effective conservation of plant genetic resources, either *in situ* or *ex situ*, and the development of applicable conservation methods need a profound scientific and technical basis. Guidelines for genetic resource collection, evaluation, and selective breeding should be defined against the background of taxonomy, origin, evolution, and, especially, the genetic composition of the species of concern (Ramanatha Rao & Hodgkin, 2002). Moreover, these guidelines should consider the major drivers of genetic variation. Population (e.g. population size) or species specific (e.g. ploidy, breeding system, and connectedness) drivers reveal a direct impact on genetic variation. Indirect drivers, such as anthropogenic measures, landscape structure, climatic, edaphic, and biotic environmental conditions, vary among ecosystems and affect species individualistically (Ramanatha Rao & Hodgkin, 2002; Vellend & Geber, 2005).

Such guidelines should be determined for both *ex situ* (gene or field bank) and *in situ* (on-farm or wild) conservation measures, since genetic diversity, found in nature,

represents a resource of enormous significance (Greene et al., 2014; IUCN et al., 1980). Thus, plant material, which is extracted from natural populations and saved *ex situ* in seed banks, tissue cultures, and botanic gardens (Maxted et al., 2000), should constitute a backup for those diversity components, which might be lost in nature through environmental change (Li & Pritchard, 2009). Although *ex situ* conservation of plants dates back to the 16th century (Hurka et al., 2008), only 29 % of globally threatened plant species (IUCN, 2013) were included in ex situ conservation programmes in 2014 (Sharrock et al., 2014). Therefore, *in situ* preserved sites should be used complementarily to locate, monitor, and manage genetic diversity of natural and wild populations within defined areas for active long-term conservation (Maxted et al., 2000). 'Genetic reserves', for instance, may function as donor sites for habitat creation, restoration, or diversity enhancement providing seed material, species and habitat diversity with locally adapted, native ecotypes (Hopkins, 2009). During the last decades, *in situ* conservation methods were improved to support the dynamic conservation of plant populations (Jarvis & Hodgkin, 1999). However, even 'genetic reserves', which aim to protect the maximum range of genetic diversity with a minimal set of sites (Maxted et al., 2000), are often determined without knowledge of the genetic composition (Phillips et al., 2014). Although it is impossible to protect the gene pool of a species as a whole (Maxted et al., 2000), a high proportion of a species' genetic resource could be protected by investigating and considering its genetic composition.

Box 1.1: Calcareous grasslands, oat-grass meadows, and litter meadows as model ecosystems – Characteristics and threats

Three Natura 2000 priority habitat types (i) calcareous grasslands[1], (ii) oat-grass meadows[2], and (iii) litter meadows[3] (Figure 1.3) appeared as promising model ecosystems to study plant (epi)genetic resources.

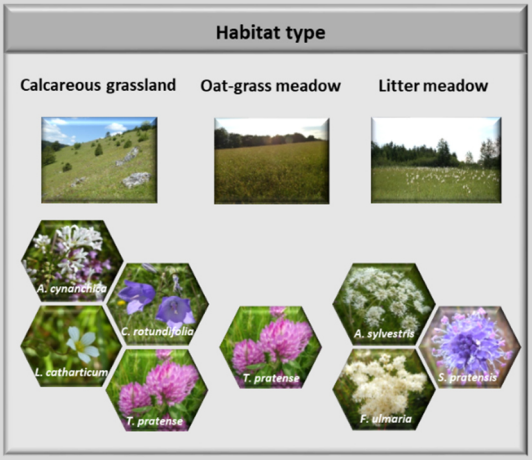


Figure 1.3: Habitat types and study species investigated in this thesis.

Calcareous grasslands above water-permeable limestone are characterized by steep slopes, shallow soils and thus, relative dry soil conditions (Wilmanns, 1955). Their existence dates back to the Neolithic (Dutoit et al., 2009; Kapfer, 2010) or Bronze Age (Poschlod & Baumann, 2010). Hence, they belong to the oldest semi-natural grassland habitats in Europe. Grazing related disturbance by cattle, sheep, and goats shaped their heterogeneous soil and sward structure (Kapfer, 2010; Olff & Ritchie, 1998) and turned calcareous grasslands into the most species-rich plant community in north-west Europe (WallisDeVries et al., 2002). Nowadays, calcareous grasslands are often highly fragmented due to intensification or abandonment (Steffan-Dewenter & Tscharntke, 2002). Consequently, many (often rare) calcareous grassland species are about to disappear or went extinct already (WallisDeVries et al., 2002). Moderate grazing with sheep and goats may avoid shrub encroachment, create dispersal corridors for species and thus, maintain species-rich calcareous grasslands.

Flower rich oat-grass meadows represent one of the youngest, but the most common Central European meadow type (Ellenberg, 1963; Poschlod et al., 2009). Traditional management consists of two (or three) cuttings per year and regular manure application to maintain productivity despite constant biomass removal (Poschlod, 2017; Poschlod et al., 2009). Their vegetation structure is even, since mowing affects all species simultaneously (Ellenberg, 1996). During the last decades, species-rich oat-grass meadows declined due to intensified management with early mowing in spring, mineral fertilizer application, and thus, increased cutting numbers (Janssens et al., 1998; Kapfer, 2010). Abandonment, ploughing, and reforestation reveal further threats (Austrheim et al., 1999; Bastian, 2013; Critchley et al., 2002). A return to traditional meadow management with adjusted cutting times and extensive manure application may counteract this decline.

The need for straw, used as bedding in stables, led to the creation of litter meadows during the 18th and 19th century (Poschlod, 2017; Poschlod & Biewer, 2005). The comparably young grassland habitats were established from fodder meadows or large wet- and peatlands on alkaline or acidic (periodically) wet sites (Poschlod & Biewer, 2005). During the last decades, bedding in stables was replaced by slatted floors (Poschlod et al., 2009) and comparably cheap mineral fertilizer allowed the transformation of unproductive litter meadows into yield-rich fodder meadows (Poschlod, 2017). These changes made the cultivation of litter meadows redundant and thus, remaining sites are threatened by drainage, intensification, abandonment, and habitat fragmentation today (Billeter et al., 2002). Maintaining a sufficient groundwater level and mowing once a year in late autumn may preserve species-rich litter meadows from extinction.

[1] 6210: Semi-natural dry grasslands and scrubland facies on calcareous substrates – *Xerobromion and Mesobromion*

[2] 6510: Lowland hay meadows - *Arrhenatherion*

[3] 6410: Molinia meadows on calcareous, peaty or clayey-silt-laden soils - *Molinion caeruleae*

[Source: https://www.bfn.de/en/activities/natura-2000/habitat-types-and-species/natura-2000- habitats-in-germany.html/ Applied 20 December 2019]

Thesis outline

The Convention on Biological Diversity (CBD) has emerged as key driver in environmental policy since 1992 (Hopkins, 2009). The alarming biodiversity decline during the last decades (Hallmann et al., 2017; Pimm et al., 2014; Seibold et al., 2019) made the protection of biodiversity more than ever to an issue of regional and global security. In Europe, semi-natural grasslands constitute the most diverse ecosystems hosting nearly one fifth of Europe's endemic vascular plant species (Hobohm & Bruchmann, 2009) and a large proportion of the vertebrate and invertebrate fauna (Hopkins & Holz, 2006). Moreover, they provide a great amount of ecosystem services (Hopkins, 2009). Semi-natural grassland habitats and some of their characteristic species are already protected by the Habitats Directive (COM, 1992), but comprehensive conservation of plant genetic resources is still in its infancy.

A clear understanding of genetic variation patterns is inevitable against the background of effective plant genetic resource conservation, e.g. in *in situ* plant genetic reserves. Therefore, the aim of this thesis was to answer the following questions: (i) What are the key drivers of genetic variation patterns in semi-natural grasslands? (ii) Is it possible to protect plant genetic resources above species level? (iii) What is the impact of the underlying habitat type? (iv) Is epigenetic variation affected by similar key drivers? (v) Is genetic and epigenetic variation interdependent?

Neutral molecular markers constitute a suitable tool to study gene flow and genetic drift. Therefore, amplified fragment length polymorphism (AFLP) analyses were applied to investigate selection neutral processes shaping the genetic variation of six calcareous grassland and litter meadow species and thus, to identify potential drivers of genetic variation. In contrast to previous studies about genetic variation and possible determinants (Falińska et al., 2010; Hensen & Wesche, 2006; Last et al., 2013; Münzbergová et al., 2013; Prentice et al., 2006; Reisch & Poschlod, 2009), our investigations were based on a multi-species approach. The study of various plant species, which are characteristic for one habitat type, revealed important insights in the underlying processes driving genetic variation of these species and of their habitats. Moreover, a multi-layer approach addressed various potential drivers of genetic variation simultaneously. Thus, the

concurrent study of land use history, surrounding landscape structure, local habitat quality, as well as population size may allow the identification of the key components driving genetic variation in semi-natural grassland plant species.

Nowadays, environmental conditions continuously change and plant species need to react immediately to survive especially challenging environmental conditions. Cytosine methylation provides a fast and valuable tool for plant species to regulate transposon silencing and gene expression without changing the underlying genetic code. Therefore, methylation-sensitive amplification polymorphism (MSAP) analyses were conducted to compare metastable, but heritable DNA methylation patterns in calcareous grassland and oat-grass meadow populations. AFLP analyses were applied, moreover, to enable a comparison between the genetic and epigenetic composition of the study species. In contrast to previous studies about (epi)genetic variation and possible determinants, this study includes a comprehensive analysis of habitat specific on-site environmental conditions to address their impact on (epi)genetic variation.

Finally, the results of this thesis were placed in the context of *in situ* plant genetic resource conservation. Strengths, limitations, and perspectives of the analyses were highlighted and further research approaches were suggested to facilitate efficient identification, establishment, and maintenance of future *in situ* plant genetic reserves.

CHAPTER 2

THE IMPACT OF HABITAT AGE, LANDSCAPE STRUCTURE, HABITAT QUALITY, AND POPULATION SIZE ON THE GENETIC VARIATION OF TYPICAL CALCAREOUS GRASSLAND PLANT SPECIES

Theresa Anna Lehmair, Ellen Pagel, Peter Poschlod, and Christoph Reisch

Calcareous grassland (No. 01) near Bichishausen, Germany

Abstract

Land use change caused an ongoing decline of calcareous grasslands throughout Europe during the last decades. Subsequent habitat deterioration affects not only species diversity, but also the genetic variation of these species. Thus, the aim of our study was to identify the drivers of genetic variation in common calcareous grassland plant species. More specifically, we tested whether genetic diversity or differentiation of *Asperula cynanchica*, *Campanula rotundifolia*, and *Linum catharticum* depend on habitat age, landscape structure, habitat quality, and/or population size.

In our study we observed no significant influence of habitat age on genetic diversity and differentiation. Habitat quality also had no impact on genetic diversity and population size only showed weak effects. However, genetic diversity strongly depended on landscape structure represented by distance to the nearest settlement, total area of calcareous grasslands, and their connectivity.

Since landscape structure is indirectly shaped by land use, our study supports the observation that genetic variation is strongly affected by grazing patterns. Thus, moderate grazing intensities over long time seem to increase levels of genetic diversity, which in turn suffers from periods of overgrazing or abandonment.

Key words

AFLP; calcareous grassland; genetic variation; *Asperula cynanchica*; *Campanula rotundifolia*; *Linum catharticum*

Introduction

Central European calcareous grasslands may apply as local biodiversity hotspots due to their long existence, habitat diversity, and species richness (Karlik & Poschlod, 2009; Poschlod, 2017; Steffan-Dewenter & Tscharntke, 2002). More precisely, they represent valuable habitats for many specialised, rare, and endangered plant or insect species and are, therefore, considered as key areas for biodiversity conservation in agricultural landscapes (Raatikainen et al., 2009; Rosengren et al., 2013).

The shift from traditional to modern (animal) husbandry caused a drastic decline of calcareous grasslands during the last 150 years (Poschlod, 2017; WallisDeVries et al., 2002). Due to abandonment and intensification more than 70 % of the calcareous grasslands on the Swabian Alb in south-west Germany disappeared until the 1990s (Mattern et al., 1992; Steffan-Dewenter & Tscharntke, 2002). Remnant calcareous grasslands are often highly fragmented and small in size. Populations in these habitat patches may consequently suffer from reduced probabilities of gene flow and increased genetic drift (Aguilar et al., 2008). Therefore, habitat loss affects not only biodiversity at the species level, but also the genetic variation of local plant populations (Ouborg et al., 2006). Following May (1994), genetic variation represents the most fundamental level of biodiversity. Levels of genetic variation are shaped by changing environmental conditions driving natural selection, adaptation, gene flow, genetic drift, and stochastic processes (McKay et al., 2005; Rosengren et al., 2013). To protect biodiversity fundamentally, we need to identify the key variables influencing genetic variation.

Calcareous grasslands are characterized by a diverse land use history as well as management continuity and could, therefore, be found either on historically old (‘ancient’) or historically young (‘recent’) sites. Populations on sites with different habitat age may show comparable genetic variation levels if gene flow is high at the time of founding and afterwards (Vandepitte et al., 2010). Nevertheless, the genetic variation of populations on recent sites seems to depend on both the number and origin of colonists (Wade & McCauley, 1988) as well as the rate of gene flow and selection after colonization (Barrett et al., 2008). These populations may, therefore, show reduced genetic variation by bottlenecks and increased divergence among populations by selection (Dlugosch & Parker,

2008; Wade & McCauley, 1988). Due to potential past and present bottleneck, selection, or gene flow events, we would expect an impact of habitat age on the genetic variation of typical calcareous grassland species.

Past and present landscape structures provide valuable information about potential gene flow and further dispersal processes (Prentice et al., 2006; Purschke et al., 2012). The impact of both habitat size and area of surrounding habitats on biodiversity was analysed for many species groups and habitats, since MacArthur and Wilson (1967) established the theory of island biogeography. Hence, various studies reported that plant populations on small and isolated calcareous grasslands, with reduced gene flow, increased inbreeding as well as genetic drift, showed reduced seed set (Kéry et al., 2000), genetic erosion (Honnay et al., 2007) and finally higher extinction risks (Spielman et al., 2004). Besides habitat size, habitat connectivity and the kind of grazing management supply essential information about possible gene flow and seed dispersal in networks of (fragmented) habitat patches (Reitalu et al., 2010). Due to rescue effects, highly connected sites are expected to show increased colonisation and reduced extinction rates (Brown & Kodric-Brown, 1977). Additionally, grazing, e.g. by sheep (typically for calcareous grasslands), ensures propagule dispersal over large distances and improves habitat quality by trampling and browsing (Fischer et al., 1996; Willerding & Poschlod, 2002). Thus, it can be hypothesized that surrounding landscape structures and resulting gene flow mechanisms are important determinants for genetic variation in highly diverse calcareous grasslands.

The abandonment of migratory sheep farming and thereby lower grazing pressure on calcareous grasslands led to deteriorated habitat conditions in the last decades (Zulka et al., 2014). The missing removal of biomass resulted in litter accumulation, eutrophication, and thus, increasing vegetation height with grasses dominating (Jacquemyn et al., 2011). The germination of calcareous grassland species depends on the availability of light and open soil (Grubb, 1977). Moreover, thick litter layers acting as seed traps (Ruprecht & Szabó, 2012) and high vegetation causing ground shadowing (Jensen & Gutekunst, 2003) inhibit germination and establishment of these species. Therefore, an impact of the local vegetation structure, which is also an indicator for habitat quality, on genetic variation can be expected.

Despite intact habitat quality, habitat fragmentation could lead to isolated populations with decreased population size. Small populations may react more sensitive to demographic and environmental changes due to the fixation of deleterious alleles by genetic drift (Young et al., 1996). These populations will show lower genetic variability, consequently increased levels of inbreeding (Van Treuren et al., 2005), and therefore, face a higher risk of extinction (Ouborg et al., 2006; Spielman et al., 2004). Many empirical studies observed a positive impact of population size on the genetic variation of calcareous grassland species (Leimu et al., 2006) and thus, we predict a positive association between population size and genetic variation.

Considering all these aspects, the aim of this study was to disentangle the relative impact of abiotic factors on the genetic variation of common calcareous grassland species. In changing environments, gene flow, migration, and/or dispersal potential of species may be represented by different levels of genetic variation (Holderegger et al., 2006). Thus, we asked the following questions: (i) Is genetic diversity influenced by habitat age? Are populations of different habitat age genetically differentiated? (ii) What is the impact of past and/or present landscape structure on genetic diversity? (iii) Is genetic diversity affected by the present habitat quality and/or population size?

Methods

Study design

For our study, we selected 19 calcareous grasslands all over the Swabian Alb in south-west Germany (Figure 2.1, Table S2.1). This region belongs to the largest Jurassic low mountain range in Central Europe (Park, 2017). The climate is characterized by cool, humid westerly winds with an annual average temperature between 6.7 and 8.0 °C and an average precipitation from 750 to 1050 mm/year (Jooß, 2014).

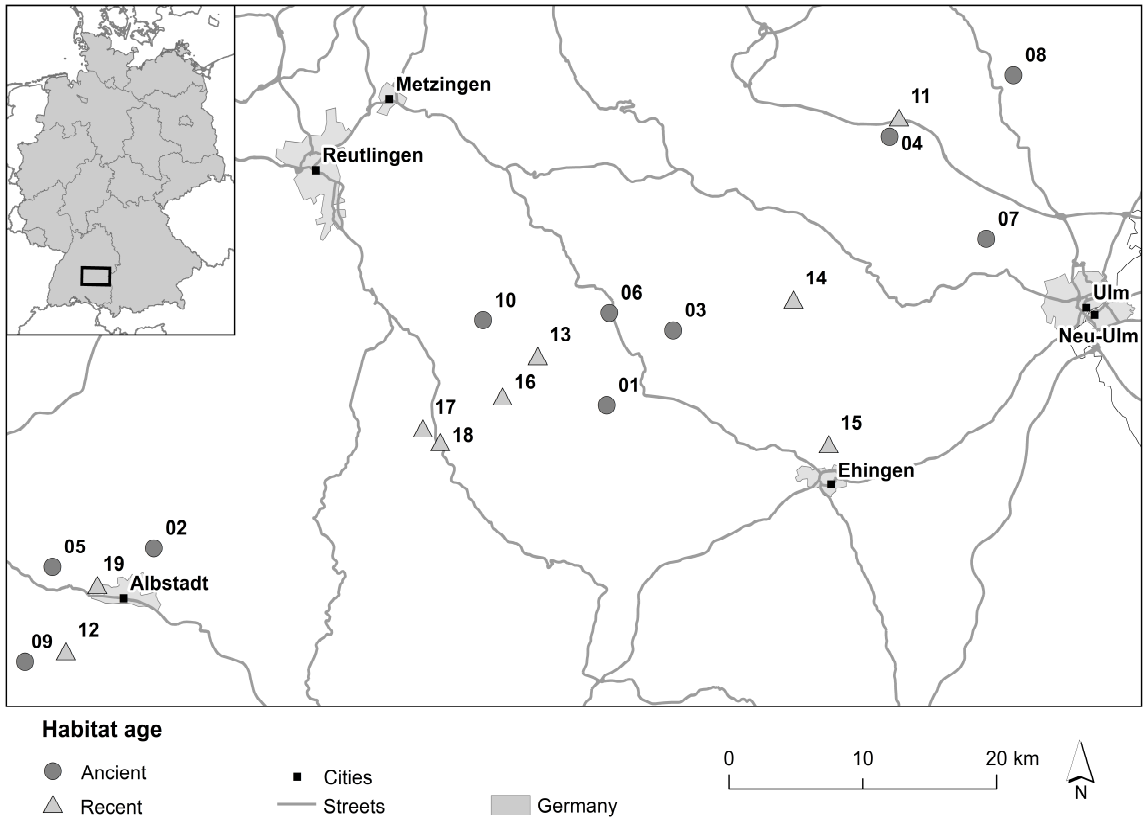


Figure 2.1: Geographic position and habitat age of the analysed *A. cynanchica*, *C. rotundifolia*, and *L. catharticum* populations.

In order to study the impact of habitat age on genetic diversity and differentiation of common calcareous grassland species, we sampled populations on sites with different habitat age (Reitalu et al., 2010). We selected ten historically old sites ('ancient sites'), which are calcareous grasslands since before the 1820s, and nine historically young sites ('recent sites'), which developed from arable fields during the 1900s (Figure 2.1). The

habitat age was determined using historical cadastral maps from 1820 to 1850 as well as 1902 to 1914. Further, topographical maps from 1951 to 1953 and actual aerial photographs were examined using the software ArcGIS® 10.3.1 (Esri, Redlands, CA, USA) (Table S2.2).

(a) **(b)** **(c)**

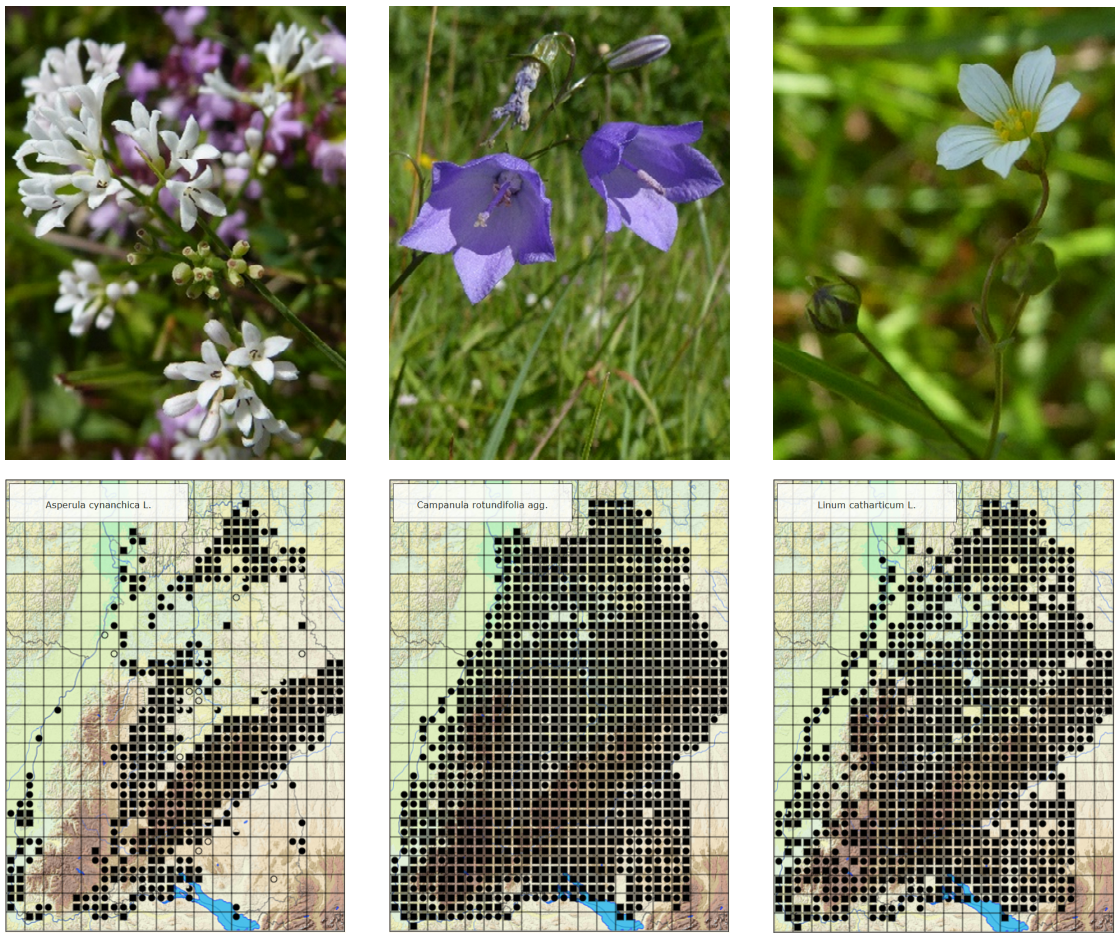


Figure 2.2: *A. cynanchica* (a), *C. rotundifolia* (b), *L. catharticum* (c) and their spatial distribution over Baden-Württemberg.
[Source: http://www.florabw.recorder-d.de/ Applied: 29 August 2019]

At each site, we analysed genetic variation of three typical calcareous grassland species: *Asperula cynanchica* L., *Campanula rotundifolia* L. s. str., and *Linum catharticum* L. (Figure 2.2 a - c). *A. cynanchica* (Rubiaceae; 2n = 22, (44)) is flowering mauve or whitish from June to September (Kühn et al., 2004). Main pollinators are insects, e.g. bees,

bumblebees, wasps, bombylides, or syrphids, but occasionally *A. cynanchica* could perform self-pollination (Kühn et al., 2004). The purple bell-shaped flowers of *C. rotundifolia* (Campanulaceae; 2n = 34, 68) are mostly pollinated by bees between June and October (Kühn et al., 2004). The annual *L. catharticum* (Linaceae; 2n = 16), which can sometimes live longer, is flowering white-yellowish from May to July (Kühn et al., 2004). It generally shows self-pollination, but could also be insect-pollinated by bees, bumblebees, wasps, bombylides, or syrphids (Kühn et al., 2004). All three species can be dispersed ecto- and/or endozoochorously (Poschlod et al., 2003).

To analyse the impact of landscape structure on genetic diversity, we digitized historical cadastral maps (1820 - 1850) as well as actual topographical maps (2014 - 2018) in a 3 km radius around each study site (Table S2.2). As potential explanatory variables, we identified the area of each study site (AREA_S) and measured the past and present distance to the nearest settlement (DIST_1820; DIST_2018) (Table S2.3). Additionally, we calculated the past and present total area of surrounding calcareous grasslands (AREA_1820; AREA_2018) as well as the past and present connectivity (CON_1820; CON_2018) per circle (Table S2.3). The connectivity was determined according to Hanski (1994) as $S_i = \sum_{j \neq i} \exp(-\alpha d_{ij}) A_j$ where S_i is the connectivity of the patch i, d_{ij} is the distance (km) between patches i and j, A_j is the area (ha) of the patch j, and α is the parameter of the exponential distribution setting the influence of distance on connectivity (Helm et al., 2006). Following Lindborg and Eriksson (2004) and Reitalu *et al.* (2010) α was set to one and not weighted by the dispersal abilities of the plant species in the community.

Data about the cover of vascular plants, mosses, litter, and open soil were incorporated per study site to investigate the influence of habitat quality on genetic diversity (Table S2.4). Furthermore, population size was determined by counting the number of individuals in 10 to 15 1 m^2 plots in the field. The average number of individuals per square metre was then multiplied with AREA_S (Reisch et al., 2018) (Table S2.4). For those study sites, where no individual could be found in the 1 m^2 plots although plant material was collected, the total number of individuals was set from 0 to 1 before multiplying.

For molecular analyses we took leaf samples from 16 individuals per population and species to cover more than 90 % of the total genetic diversity (Leipold et al., 2020).

Molecular analyses

DNA extraction was conducted following the CTAB protocol from Rogers and Bendich (1994) modified by Reisch (2007). DNA quality and concentration were determined with a spectrophotometer. All DNA samples were diluted to the same level of 7.8 ng DNA per µl H_2O. Genetic variation within populations was determined for 912 individuals using genome-wide genotyping with amplified fragment length polymorphisms (AFLP; Vos *et al.* 1995). The AFLP analyses were performed following the standardized protocol of Beckmann Coulter (Bylebyl et al., 2008; Reisch, 2008). We screened 36 primer combinations per species to choose three appropriate primer combinations for the selective amplification (Table S2.5). An automated capillary electrophoresis machine (GeXP, Beckmann Coulter) was used to separate the fluorescence-labelled DNA fragments by capillary gel electrophoresis. Fragment data were analysed manually applying the software Bionumerics 4.6 (Applied Maths, Kortrijk, Belgium). Only strong and clearly defined fragments were taken into account for further analyses, while samples without clear banding pattern were repeated.

The reproducibility of the AFLP analyses was tested by calculating the genotyping error rate (Bonin et al., 2004). Therefore, 10 % of all analysed samples were replicated twice and the percentage of fragments with differences between original and replicate was evaluated. The genotyping error rates of *A. cynanchica*, *C. rotundifolia,* and *L. catharticum* were 2.6 %, 4.2 %, and 2.5 % respectively.

Statistical analyses

Binary (0/1) matrices were created applying Bionumerics 4.6. Using this matrices, genetic diversity within each population was calculated as Nei's gene diversity (GD) $H = 1 - \sum(p_i)^2$, with p_i representing the allele frequency, in PopGene 32 (Yeh et al., 1997). We calculated a Kruskal-Wallis test with a post hoc Dunn's test and a Bonferroni p-adjustment in R (R Core

Team, 1978) to compare Nei's gene diversity on species level and to test the dependence of Nei's gene diversity on habitat age.

Hierarchical analyses of molecular variance, AMOVA, based on pairwise Euclidian distances among samples, were conducted applying the software GenAlEx 6.41 (Peakall & Smouse, 2006). Thus, the genetic variation within and among populations as well as among populations of different habitat age was analysed.

Mantel tests with 999 permutations were calculated using GenAlEx 6.41 (Peakall & Smouse, 2006) to test the correlation between geographic and genetic distances (Φ_{PT} values calculated in the AMOVA) among populations (Mantel, 1967).

We then built a starting model with the full set of scaled and centred explanatory variables (except for habitat age) to analyse the relationship between genetic diversity and potential explanatory variables. More specifically, we formulated linear regression models for each species in R (R Core Team 1978) to describe the variation of Nei's gene diversity related to (i) habitat age, (ii) AREA_S, (iii) AREA_1820, (iv) AREA _2018, (v) CON_1820, (vi) CON_2018, (vii) DIST_1820, and (viii) DIST _2018, which were described above. Further data about the coverage of (ix) vascular plants, (x) mosses, (xi) litter, and (xii) open soil, as well as the (xiii) population size of each species were included per study site. The impact of those variables on the variation of the mean Nei's gene diversity over all species was tested in an additional model. We then ranked all potential linear models according to AICc values (Akaike Information Criterion corrected for small sample sizes) to detect the models with the highest information content (Burnham & Anderson, 2002). Differences between past and present landscape variables (Table S2.6) were tested by calculating Wilcoxon-Mann-Whitney tests. Correlations among the explanatory variables (ii – xiii) were analysed with correlation tests (Pearson correlation coefficients) (Table S2.7).

Results

AFLP analyses resulted in 148, 151, and 146 fragments for *A. cynanchica*, *C. rotundifolia*, and *L. catharticum*. 69.59 %, 68.49 %, and 44.81 % of these fragments were polymorphic. No identical genotypes were detected.

Nei's gene diversity of *A. cynanchica* populations ranged between 0.21 and 0.30 (GD_{mean} = 0.27) (Table 2.1). *C. rotundifolia* populations showed with 0.24 a lower mean Nei's gene diversity than *A. cynanchica* ($p = 0.054$) (Figure 2.3 a). It ranged from 0.22 to 0.26 (Table 2.1). *L. catharticum* populations indicated a significantly lower mean Nei's gene diversity (GD_{mean} = 0.16) than *A. cynanchica* ($p < 0.001$) and *C. rotundifolia* ($p < 0.001$) (Figure 2.3 a). The lowest value was 0.13 and the highest 0.19 (Table 2.1). Mean Nei's gene diversity over all analysed species (GD_{mean} = 0.22) ranged from 0.20 to 0.24 (Table 2.1). AMOVAs (Table 2.2) indicated only weak levels of differentiation among populations of *A. cynanchica* (Φ_{PT} = 0.072), *C. rotundifolia* (Φ_{PT} = 0.048), and *L. catharticum* (Φ_{PT} = 0.078).

Moreover, AMOVAs revealed no differentiation among populations on ancient and recent grasslands (Table 2.2). Furthermore, genetic diversity did not differ significantly among populations on ancient and recent sites (Figure 2.3 b).

Our study indicated a significant decline of the total area of calcareous grasslands, their connectivity, and their distance to the nearest settlement between the 1820s and 2018 (Table S2.6).

Mantel tests revealed significant correlations between pairwise genetic and geographic distances for *A. cynanchica* ($r = 0.41$; $p = 0.001$) (Figure 2.4 a) and *C. rotundifolia* ($r = 0.37$; $p = 0.001$) (Figure 2.4 b), but not for *L. catharticum* ($r = 0.06$; $p = 0.263$) (Figure 2.4 c).

The AICc model selection generated different linear models per species (Table 2.3 a - d). Nei's gene diversity of *A. cynanchica* populations was positively associated with CON_2018 ($p < 0.001$). Nevertheless, AREA_1820 ($p = 0.019$), DIST_2018 ($p < 0.001$), and the species' population size ($p < 0.001$) displayed a negative impact (Table 2.3 a). Genetic diversity of *C. rotundifolia* populations increased with rising DIST_1820 ($p = 0.022$) (Table 2.3 b), while Nei's gene diversity of *L. catharticum* populations was positively linked

to AREA_2018 (p = 0.021) (Table 2.3 c). Additionally, AREA_2018 showed a positive impact on the mean Nei's gene diversity over all analysed species (p = 0.003) (Table 2.3 d).

Table 2.1: Number (N) of investigated individuals per population (No.) and mean Nei's gene diversity within populations of *A. cynanchica*, *C. rotundifolia*, *L. catharticum*, and over all analysed species (All species).

		Nei's gene diversity			
No.	**N**	***A. cynanchica***	***C. rotundifolia***	***L. catharticum***	**All species**
01	16	0.288	0.246	0.163	0.232
02	16	0.296	0.243	0.179	0.239
03	16	0.256	0.218	0.172	0.216
04	16	0.290	0.257	0.154	0.233
05	16	0.285	0.254	0.187	0.242
06	16	0.261	0.227	0.177	0.222
07	16	0.272	0.229	0.141	0.214
08	16	0.275	0.227	0.154	0.219
09	16	0.267	0.239	0.147	0.218
10	16	0.228	0.240	0.142	0.203
11	16	0.288	0.254	0.166	0.236
12	16	0.304	0.229	0.174	0.236
13	16	0.270	0.243	0.139	0.218
14	16	0.260	0.254	0.187	0.234
15	16	0.243	0.231	0.131	0.202
16	16	0.240	0.236	0.128	0.201
17	16	0.248	0.258	0.133	0.213
18	16	0.210	0.253	0.150	0.204
19	16	0.280	0.224	0.130	0.211
Mean		**0.266**	**0.240**	**0.155**	**0.221**
SE		± 0.006	± 0.003	± 0.005	± 0.003

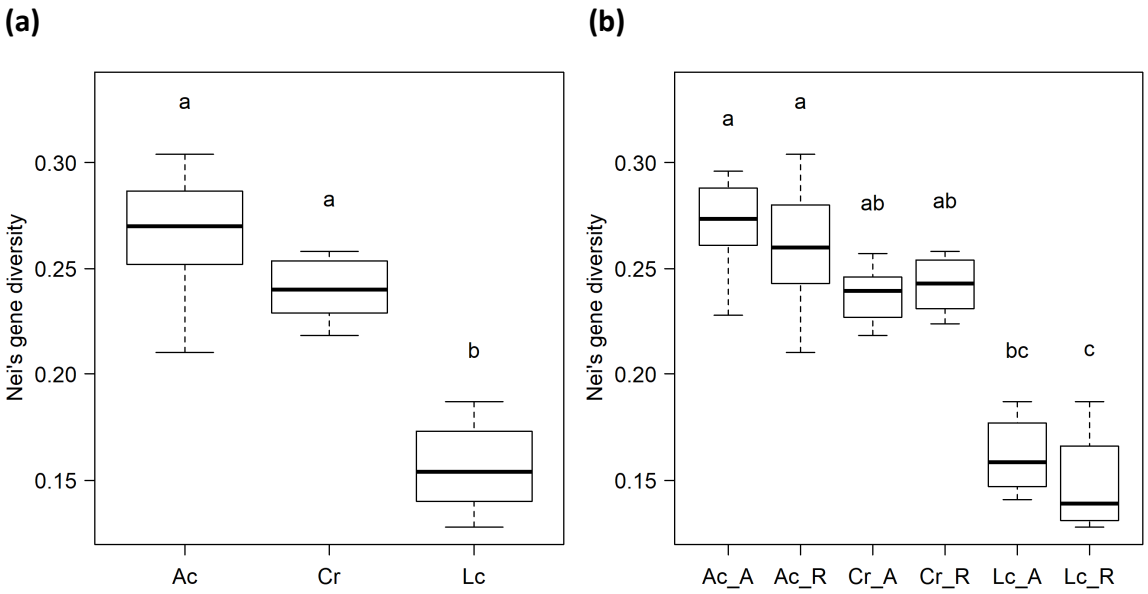


Figure 2.3: Nei's gene diversity (a) and Nei's gene diversity per habitat age (b; A: ancient; R: recent) of *A. cynanchica* (Ac), *C. rotundifolia* (Cr), and *L. catharticum* (Lc). The results of the Kruskal-Wallis test are indicated by the letters above the boxplots.

Table 2.2: Genetic variation per species among populations with different habitat age, among and within studied populations detected by AMOVA. Levels of significance are based on 999 iteration steps.

Species	AMOVA	df	SS	MS	Est. Var.	%	Φ_{PT}	
A. cynanchica	Among habitat age	1	34.15	34.15	0.00	0	0.072	***
	Among populations	17	698.70	41.10	1.45	7		
	Within populations	285	5115.19	17.95	17.95	93		
C. rotundifolia	Among habitat age	1	32.26	32.26	0.00	0	0.048	***
	Among populations	17	589.23	34.66	0.97	5		
	Within populations	285	5453.25	19.13	19.13	95		
L. catharticum	Among habitat age	1	19.09	19.09	0.00	0	0.078	***
	Among populations	17	449.13	26.42	0.97	8		
	Within populations	285	3116.63	10.94	10.94	92		

Signif. code: $p \leq 0.001$ ***

df, degree of freedom; SS, sum of squares; MS, mean squares; Est. Var., estimated variation; %, proportion of genetic variation; Φ_{PT}, indicator for genetic differentiation among populations

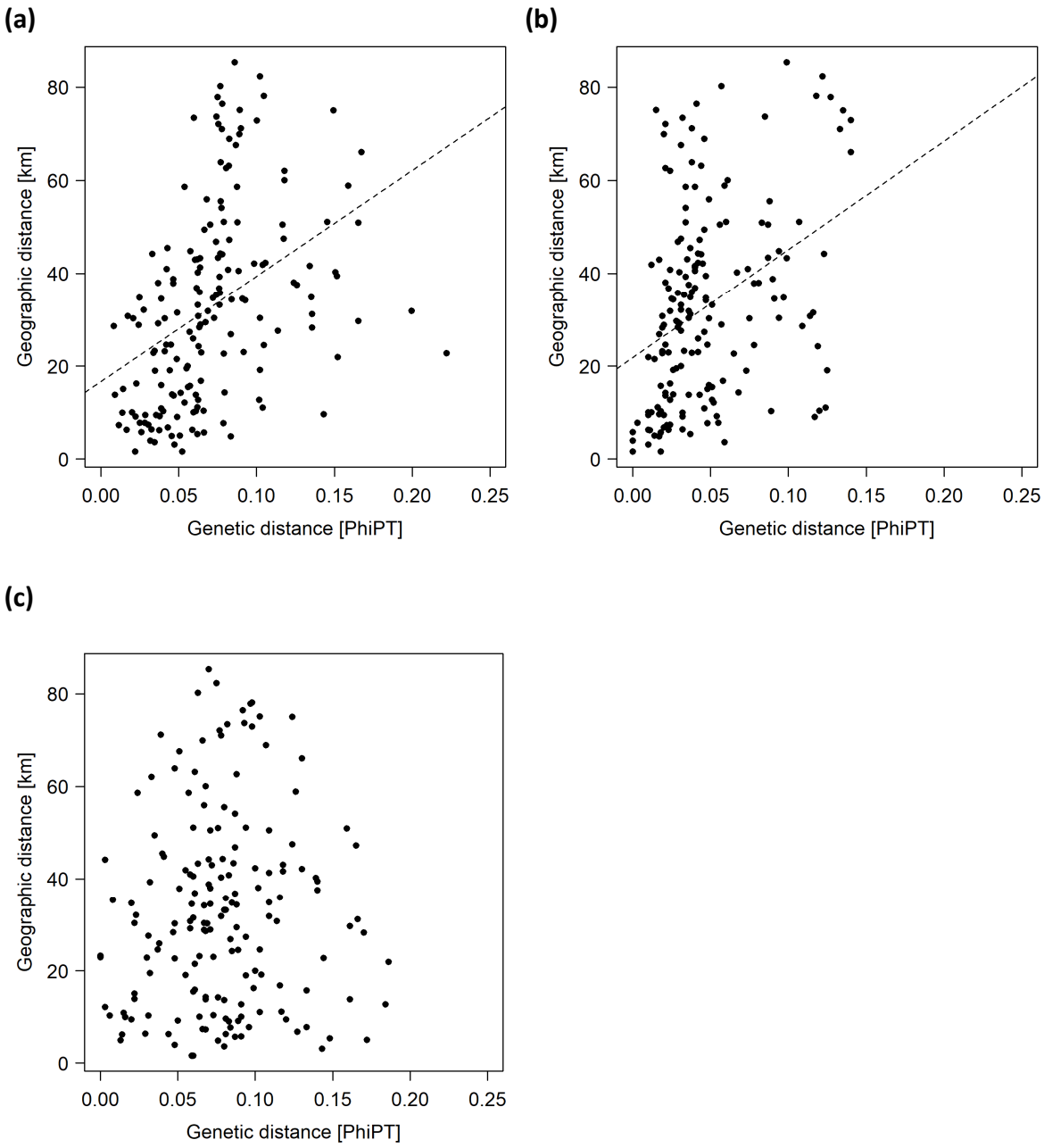


Figure 2.4: Correlation of genetic distance (Φ_{PT}) and geographic distance (km) (Manteltest) among the studied populations of *A. cynanchica* (a; r = 0.41; p = 0.001), *C. rotundifolia* (b; r = 0.37; p = 0.001), and *L. catharticum* (c; r = 0.06; p = 0.263).

Table 2.3: Linear models explaining genetic diversity patterns in *A. cynanchica* (a), *C. rotundifolia* (b), *L. catharticum* (c), and mean of all analysed species (d). The estimate, the standard error, and the p-value are given.

(a) *A. cynanchica*

		Estimate	**Std. Error**	**p-value**	
	(Intercept)	0.2664	0.00176	< 0.001	***
Response variable	**Explanatory variable**				
Nei's gene diversity	CON_2018	0.0206	0.00219	< 0.001	***
	DIST_2018	- 0.0114	0.00201	< 0.001	***
	Population size	- 0.0052	0.00194	0.019	*
	AREA_1820	- 0.0132	0.00212	< 0.001	***

Residual standard error: 0.007657 on 14 degrees of freedom
Multiple R-squared: 0.9243, Adjusted R-squared: 0.9027
F-statistic: 42.75 on 4 and 14 DF, p-value: 1.061e-07

(b) *C. rotundifolia*

		Estimate	**Std. Error**	**p-value**	
	(Intercept)	0.2401	0.00253	< 0.001	***
Response variable	**Explanatory variable**				
Nei's gene diversity	DIST_1820	0.0114	0.00450	0.022	*

Residual standard error: 0.01102 on 17 degrees of freedom
Multiple R-squared: 0.2736, Adjusted R-squared: 0.2309
F-statistic: 6.404 on 1 and 17 DF, p-value: 0.02155

(c) *L. catharticum*

		Estimate	**Std. Error**	**p-value**	
	(Intercept)	0.1555	0.00398	< 0.001	***
Response variable	**Explanatory variable**				
Nei's gene diversity	AREA_2018	0.0001	0.00005	0.021	*

Residual standard error: 0.01733 on 17 degrees of freedom
Multiple R-squared: 0.2771, Adjusted R-squared: 0.2345
F-statistic: 6.515 on 1 and 17 DF, p-value: 0.0206

(d) All species

		Estimate	Std. Error	p-value	
	(Intercept)	0.2206	0.00245	< 0.001	***
Response variable	**Explanatory variable**				
Nei's gene diversity	AREA_2018	0.0001	0.00003	0.003	**

Residual standard error: 0.01067 on 17 degrees of freedom

Multiple R-squared: 0.4116, Adjusted R-squared: 0.377

F-statistic: 11.89 on 1 and 17 DF, p-value: 0.003068

Signif. codes: p ≤ 0.001 ***; 0.001 < p ≤ 0.01 **; 0.01 < p ≤ 0.05 *

AREA_1820/AREA_2018, past and present total area of calcareous grasslands [ha]; DIST__1820/DIST_2018, past and present distances to the nearest settlement [km]; CON_2018, present connectivity

Discussion

Genetic variation

Mean genetic diversity of our study species *A. cynanchica*, *C. rotundifolia*, and *L. catharticum* complied with the genetic diversity previously reported for common grassland species (Reisch & Bernhardt-Römermann, 2014). Generally, genetic diversity depends on a species' pollination and mating system (Schoen & Brown, 1991). Therefore, the insect pollinated, outcrossing species *A. cynanchica* and *C. rotundifolia* revealed significantly higher genetic diversity levels than *L. catharticum*, which is mostly considered as self-pollinated species (Kühn et al., 2004).

Weak levels of differentiation and comparatively low Φ_{PT} values among populations led to the assumption that the spatial distance among populations (< 100 km) still seems to allow sufficient gene flow (Neel, 2008).

Habitat age

Our study revealed similar levels of genetic diversity concerning habitat age. Following Rosengren et al. (2013) the genetic diversity of recent sites may be increased if they are connected to continuously grazed ancient sites with a diverse gene pool. Therefore, sufficient gene flow at the time of founding and afterwards might reduce the effects of habitat age (Vandepitte et al., 2010).

Furthermore, we observed no significant differentiation among populations on ancient and recent grassland sites. Genetic differentiation is often described as a direct function of dispersal (Oostermeijer et al., 1996). More than 50 % of a local species pool could be transported by one sheep during a vegetation period (Fischer et al., 1996). Thus, especially dispersal by sheep is thought to have a detectable effect on the genetic variation of grazed calcareous grassland populations (Rico et al., 2014a, 2014b; Willerding & Poschlod, 2002). The suggested dispersal rate of 660,000 diaspores per 400-head sheep flock (Willerding & Poschlod, 2002) results in a substantial gene flow over long time periods and large distances (Fischer et al., 1996; Poschlod, 2017; Poschlod et al., 1998). Furthermore, hayseed of populations on ancient sites was used to establish calcareous grasslands artificially on abandoned arable fields until the 20th century (Poschlod &

WallisDeVries, 2002). According to the migrant pool model (Wade & McCauley, 1988), genetic divergence could only occur if the number of colonists is less than twice the number of migrants.

Anthropogenic land use allows comparatively high levels of gene flow (Neel, 2008) and could, therefore, establish viable populations in a relatively short time by overcoming pronounced founder effects (Helsen et al., 2013). By this means, anthropogenic land use may have led to similar diversity levels as well as undetectable genetic differentiation among populations of different habitat age.

Landscape structure

During the last century, land use change caused a quantitative decline of semi-natural grasslands, especially of calcareous grasslands (Poschlod et al., 2005). More particularly, settlement expansion (Poschlod, 2017) or abandonment of migratory sheep farming caused a massive habitat loss (WallisDeVries et al., 2002). In accordance, the present study revealed a significant decline of the total area of calcareous grasslands, their connectivity, and their distance to the nearest settlement between the 1820s and 2018.

However, CON_2018 appeared as the only positive explanatory variable for the genetic diversity of the analysed *A. cynanchica* populations. This result is corroborated by the findings of Raatikainen and Heikkinen (2009), although other studies revealed only an influence of the past connectivity on grassland species (Helm et al., 2006; Lindborg & Eriksson, 2004). The model indicates the presence of a rescue effect (Brown & Kodric-Brown, 1977). Thus, populations in small habitat patches could not only persist with a high probability (Helm et al., 2006), they even show increased genetic diversity if they are well connected.

In general, the distance to the nearest settlement and the area of surrounding calcareous grasslands may describe the movement patterns of livestock (Reitalu et al., 2010), since migratory sheep herding was the main land use in calcareous grasslands of the study region. Migratory sheep herding represents both an important vector for seed dispersal (Fischer et al., 1996; Willerding & Poschlod, 2002) and ecological disturbance by grazing and trampling (Olff & Ritchie, 1998). Thus, overgrazing may lead to increased levels

of gene flow and disturbance, while abandonment of migratory sheep herding is expected to reduce levels of gene flow as well as the probability of seedling establishment due to a thickening litter and vegetation layer (Ruprecht & Szabó, 2012). Intermediate levels of gene flow may reveal a positive impact on genetic diversity levels, while 'too low' and even 'too high' levels of gene flow may promote outbreeding depression and/or genetic 'swamping' (Bradshaw, 1984). Overgrazing and abandonment of migratory sheep herding may, therefore, decrease both species (Klimek et al., 2007) and genetic diversity.

Unexpectedly, *C. rotundifolia* populations showed a positive impact of DIST_1820 on Nei's gene diversity, resulting in decreased levels of genetic diversity around settlements. Therefore, we assume that grasslands close to those settlements may reflect the impact of periodic overgrazing with increased levels of gene flow and disturbance during the 1820s.

Nevertheless, the linear model for *A. cynanchica* displayed a negative impact of DIST_2018. Thus, the highest levels of genetic diversity occurred in populations near settlements in 2018. Despite nowadays ongoing decline of livestock grazing (Poschlod, 2017), we suggest that grazing intensity and associated gene flow is still at an intermediate level around present settlements. Like Reitalu et al. (2010), we found an unimodal association between genetic diversity and the distance to the nearest settlement. The authors interpreted this result in terms of the intermediate disturbance hypothesis of Connell (1978) on a landscape scale. In calcareous grasslands, the landscape scale corresponds to the intensity of grazing and associated disturbance. Therefore, these results could also be explained in terms of a classic intermediate disturbance hypothesis (Connell, 1978).

Unexpectedly, the linear model for *A. cynanchica* displayed a negative impact of AREA_1820 on genetic diversity. This idiosyncratic result is inconsistent with the generally accepted expectation that populations, which are embedded in a landscape matrix containing a large proportion of grasslands, are more likely to reveal high levels of genetic diversity (Rosengren et al., 2013). Therefore, we suggest that comparatively high levels of gene flow in the past may have led to a highly unified and impoverished gene pool. Moreover, *A. cynanchica* populations could have also been affected by periodic

overgrazing with increased levels of disturbance during the 1820s. Thus, *A. cynanchica* still seems to suffer from a kind of 'over-connection' with comparatively high measures of gene flow and/or increased levels of disturbance by grazing animals during the 1820s.

Nei's gene diversity of *L. catharticum* populations was positively associated with AREA_2018. On the one hand, small and isolated habitat fragments may show reduced fitness levels and finally extinction if they suffer from edge effects and the invasion of generalist species (Leimu et al., 2006). On the other hand, a large patch size and a high proportion of surrounding grasslands may increase the variability of the incoming gene flow (Prentice et al., 2006). Thus, the total area of surrounding grassland patches has not only a positive effect on species richness and presence (Raatikainen et al., 2009), it could also increase the genetic diversity of species (Dahlström et al., 2006). Levels of genetic diversity seem to come up with an intermediate gene flow level, since significantly lower values of AREA_2018 (compared to AREA_1820) positively affected genetic diversity in *L. catharticum*. Therefore, high genetic diversity seems to depend on an intermediate size level of surrounding calcareous grasslands or rather gene flow.

The last linear model displayed a positive effect of AREA_2018 on the mean genetic diversity of all analysed species. AREA_2018 is correlated with CON_2018, which also showed a positive impact on the genetic diversity of *A. cynanchica*. Hence, AREA_2018 influenced the genetic diversity of both *L. catharticum* and *A. cynanchica* populations and turned out as another important explanatory variable for the genetic diversity of typical calcareous grassland species.

However, following Jacquemyn et al. (2006), pollination as well as dispersal vectors determinate gene flow over great geographic distances. The analysed species revealed different isolation by distance patterns, although all three species are dispersed ecto- and/or endozoochorously (Poschlod et al., 2003), e.g. by grazing sheep. The insect pollinated perennials, *A. cynanchica* and *C. rotundifolia*, showed lower gene flow over increasing distances (isolation by distance), since pollinating insects may rarely travel distances larger than 1 km (Kwak et al., 1998; Steffan-Dewenter & Tscharntke, 2002). The mainly self-pollinated *L. catharticum* did, therefore, not reveal any isolation by distance.

Habitat quality and population size

The habitat quality showed no impact on the genetic diversity of the analysed calcareous grassland species. However, the linear model for *A. cynanchica* displayed an influence of the species' population size. Although correlations between population size and genetic diversity are generally positive (Leimu et al., 2006), the genetic diversity of *A. cynanchica* decreased with increasing population size. Grassland plant species with comparatively large population size, long life cycles, and slow intrinsic dynamics may occur as remnant populations in modern landscapes (Maurer et al., 2003). Additionally, Piqueray et al. (2011) observed that the present occurrence of species can be influenced by past habitat configuration. These species often show a time lag between habitat loss, fragmentation, and their consequences on genetic diversity (Helm et al., 2006). Various studies revealed a significant relationship between the genetic diversity and the linkage of the studied populations in the past landscape. Thus, they indicated a delayed response of genetic diversity to habitat fragmentation (Honnay et al., 2007). The total area of calcareous grasslands as well as the connectivity of the study sites significantly decreased since the 1820s. Thus, especially the huge populations of *A. cynanchica* seem to suffer from a kind of extinction debt today. We, therefore, assume that the slow response of *A. cynanchica* populations to previous habitat loss events led to decreased genetic diversity levels although the present population size is high.

Conclusions

From our study it can be concluded that habitat age seems to have no impact on genetic variation within and among populations if a sufficient number of source populations is nearby and gene flow is high. Therefore, our results support the assumption that the populations of the study species have previously been or are still connected by gene flow.

However, our study revealed a significant impact of the surrounding landscape structure and related land use patters. Thus, moderate grazing intensities over long time may lead to increased levels of genetic diversity by intermediate levels of gene flow, while periods of overgrazing or abandonment seem to result in genetically less variable plant populations.

Finally, neither habitat quality nor population size appeared as crucial variables for genetic diversity patterns in our study. These findings provide evidence that surrounding landscape patterns are more important to preserve the genetic variation of typical calcareous grassland species than local site conditions.

CHAPTER 3

GENETIC VARIATION OF LITTER MEADOW SPECIES REFLECTS GENE FLOW BY HAY TRANSFER AND MOWING WITH AGRICULTURAL MACHINES

Theresa Anna Lehmair, Ellen Pagel, Peter Poschlod, and Christoph Reisch

Litter meadow (No. 19) near Sigrazhofen, Germany

Abstract

Litter meadows, historically established for litter production, are species-rich and diverse ecosystems. These meadows drastically declined during the last decades along with decreasing litter use in modern livestock housing. The aim of our study was to identify the drivers of genetic variation in litter meadow species. Therefore, we tested whether genetic diversity and differentiation depend on habitat age, landscape structure, habitat quality, and/or population size.

We analysed 892 individuals of *Angelica sylvestris*, *Filipendula ulmaria*, and *Succisa pratensis* from 20 litter meadows across the Allgäu in Baden-Württemberg (Germany) using AFLP analyses.

All study species showed moderate levels of genetic diversity, while genetic differentiation among populations was low. Neither genetic diversity nor differentiation were clearly driven by habitat age. However, landscape structure, habitat quality as well as population size revealed different impacts on the genetic diversity of our study species. Past and present landscape structures shaped the genetic diversity patterns of *A. sylvestris* and *F. ulmaria*. The genetic diversity of *F. ulmaria* populations was, moreover, influenced by the local habitat quality. *S. pratensis* populations seemed to be affected only by population size.

All explanatory variables represent past as well as present gene flow patterns by anthropogenic land use. Therefore, we assume that genetic diversity and differentiation were shaped by both historical creation of litter meadows via hay transfer and present mowing management with agricultural machines. These land use practices caused and still cause gene flow among populations in the declining habitats.

Key words

AFLP; litter meadow; semi-natural grassland; conservation; genetic variation; management

Introduction

Litter meadows constitute valuable habitats for many specialised, rare, and endangered plant and animal species (Wheeler, 1988). Therefore, these semi-natural grasslands belong to the most species-rich ecosystems in Central Europe (Kull & Zobel, 1991) and represent key areas for biodiversity conservation in agricultural landscapes, despite their comparably short land use history and limited spatial distribution.

According to Poschlod (2017), the construction of railway lines opened up the Alpine foreland region at the end of the 19th century. Agricultural crops were imported and subsistence farming efforts became redundant. Farming practices consequently changed from laborious cultivation of arable fields to more efficient grassland management for livestock farming. During this time, straw, used as bedding in stables, became scarce. Therefore, litter meadows were established either by transforming fodder meadows or by mowing large wet- and peatlands. Whereas sowing and/or planting of litter plants were recommended for the establishment in drained ponds or peat-mined areas, Stebler (1898a) described four management treatments for the conversion of fodder meadows into litter meadows without ploughing: (i) late cutting over several years, (ii) waiver of fertilization, (iii) irrigation, and (iv) resowing seeds or planting seedlings. Moreover, litter meadows were established by hayseed application (Müller, 1752). During the 1960s, litter meadow cultivation became redundant due to massive land use changes (Poschlod, 2017). Slatted floors gained more relevance in animal husbandry and thus, liquid manure replaced solid manure as preferred fertilizer. Furthermore, mineral fertilizer became comparably cheap, leading to a transformation of unproductive litter meadows into more productive fodder meadows.

Nowadays, remaining litter meadows are threatened by land use intensification, abandonment, and habitat fragmentation (Billeter et al., 2002). Habitat fragmentation limits pollen and seed exchange, restricting gene flow among populations (Honnay et al., 2006; Schmitt, 1983; Steffan-Dewenter & Tscharntke, 1999; Willerding & Poschlod, 2002) and increasing, therefore, the likelihood of inbreeding depression, the accumulation of deleterious mutations, and the extent of genetic drift (Picó & Van Groenendael, 2007; Young et al., 1996). Consequently increased genetic differentiation and reduced genetic

diversity (Barrett & Kohn, 1991; McKay et al., 2005) may lower individual plant fitness and thus, increase their extinction risk (Ellstrand & Elam, 1993; Young et al., 1996). Hence, the knowledge about potential impact factors on genetic variation patterns becomes highly relevant to protect genetic variation as a fundamental level of biodiversity (May, 1994).

Due to an outstanding land use history, litter meadows could be found either on historically old ('ancient') or historically young ('recent') sites. In this study, ancient sites were wet grasslands at least since the 1800s, while recent sites were artificially created on drained ponds during the 1900s. High gene flow at the time of establishment and afterwards may lead to comparable levels of genetic variation among populations on sites with different habitat age (Vandepitte et al., 2010). Nevertheless, the number and origin of colonists (Wade & McCauley, 1988; Whitlock & McCauley, 1990) as well as the rate of gene flow and selection after colonization (Dlugosch & Parker, 2008) drive genetic variation patterns of populations on recent sites. These populations may, therefore, show both reduced genetic variation due to bottlenecks and increased divergence among populations by selection (Dlugosch & Parker, 2008; Wade & McCauley, 1988). Previous studies observed already comparatively decreased genetic variation levels within and among populations on recent sites (Dlugosch & Parker, 2008; Jacquemyn et al., 2004; Ramakrishnan et al., 2010). Hence, we expected an impact of habitat age on the genetic variation of typical litter meadow species.

Over the past century, biodiversity decline was mainly induced by habitat loss at local, regional, and global scales (Balmford et al., 2005). Small populations, suffering from disrupted mutualistic interactions with pollinators or seed dispersers (Tscharntke & Brandl, 2004), show enhanced extinction rates due to increased levels of inbreeding, loss of genetic variation through genetic erosion, fitness decline, and loss of evolutionary adaptation potential (Adriaens et al., 2006; Young et al., 1996). Nevertheless, rescue effects may lead to increased colonisation and reduced extinction rates in highly connected sites (Brown & Kodric-Brown, 1977). We hypothesize, therefore, an impact of habitat size and connectivity on genetic variation. Moreover, gene flow, seed dispersal and establishment are influenced by land use patterns (Purschke et al., 2012; Reitalu et al., 2010) representing further determinants for gene flow and genetic variation in today's fragmented landscapes.

Populations are sometimes affected more by historic than by present landscape configurations due to a time lag in species' response (Adriaens et al., 2006). Hence, we included past as well as present landscape structures in our analyses.

Abandonment and missing biomass removal led to deteriorated habitat conditions in litter meadows. Moss and/or litter layers build-up and act as seed traps (Ruprecht & Szabó, 2012), while increased vegetation height causes ground shadowing (Jensen & Gutekunst, 2003). Germination as well as establishment of seedlings are consequently restrained (Maas, 1988; Poschlod & Biewer, 2005; Špačková & Lepš, 2004). Populations may decrease in size and a decline of genetic variation becomes more likely (Billeter et al., 2002). Therefore, we predict an impact of habitat quality on the genetic variation of common litter meadow species.

In modern fragmented landscapes, remaining litter meadows are often small, fragmented, and isolated. Populations on these sites are comparatively small and more vulnerable to demographic and environmental stochasticity, despite intact vegetation structure (Hooftman et al., 2003). These populations may suffer from reduced probabilities of gene flow, increased genetic drift, and enhanced levels of inbreeding (Aguilar et al., 2008; Van Treuren et al., 2005). Therefore, they may show lower genetic variability, reduced generative (Schmidt & Jensen, 2000) as well as vegetative performance (de Jong & Klinkhamer, 1994), and face a higher risk of extinction (Ouborg et al., 2006; Spielman et al., 2004). Various studies observed already a positive relationship between population size and genetic variation (Leimu et al., 2006). Hence, we would expect a positive impact of population size on genetic variation as well.

A range of studies already investigated the impact of habitat age, past and present landscape structure, habitat quality, and population size on genetic variation in dry grassland habitats (e.g. Prentice et al. 2006; Schmidt et al. 2009; Baessler et al. 2010; Rosengren et al. 2013; Reisch et al. 2017). Nevertheless, studies concerning wet grassland habitats, such as litter meadows, are still scarce.

Therefore, we analysed the genetic variation of three widespread litter meadow species using amplified fragment length polymorphism (AFLP) analyses. We chose the mainly insect-pollinated perennials *Angelica sylvestris*, *Filipendula ulmaria*, and *Succisa*

pratensis (Kühn et al., 2004) as study species. We ranked linear regression models according to AICc values to shed light on the relative importance of environmental factors on genetic variation patterns of the studied litter meadow species. Hence, the land use history and thus, the habitat age of the studied litter meadows was reconstructed using historical cadastral maps from different points in time. Moreover, past and present landscape structures including distance to the nearest settlement, area size, total area of surrounding wet grasslands, and connectivity were quantified on the basis of historic (1800s) and present (2018) cadastral maps. Local habitat quality was investigated with regards to vegetation cover data and population size. Applying these methods we aimed at answering the following questions: (i) What is the impact of habitat age on genetic diversity? Are populations of different habitat age genetically differentiated? (ii) Is genetic diversity influenced by past and/or present landscape structure? (iii) How is genetic diversity shaped by present habitat quality and/or population size?

Methods

Study design

In our study, we analysed the genetic variation of three typical litter meadow species: *Angelica sylvestris* L. (Apiaceae; 2n = 22), *Succisa pratensis* MOENCH (Dipsacaceae; 2n = 18), and *Filipendula ulmaria* (L.) Maxim. (Rosaceae; 2n = 14) (Figure 3.1 a - c). *A. sylvestris* and *S. pratensis* flower between July and September, while *F. ulmaria* flowers from June to August. All study species are perennials with a mixed mating system, showing insect (e.g. bees, syrphids, wasps, and beetles) as well as self-pollination (Kühn et al., 2004).

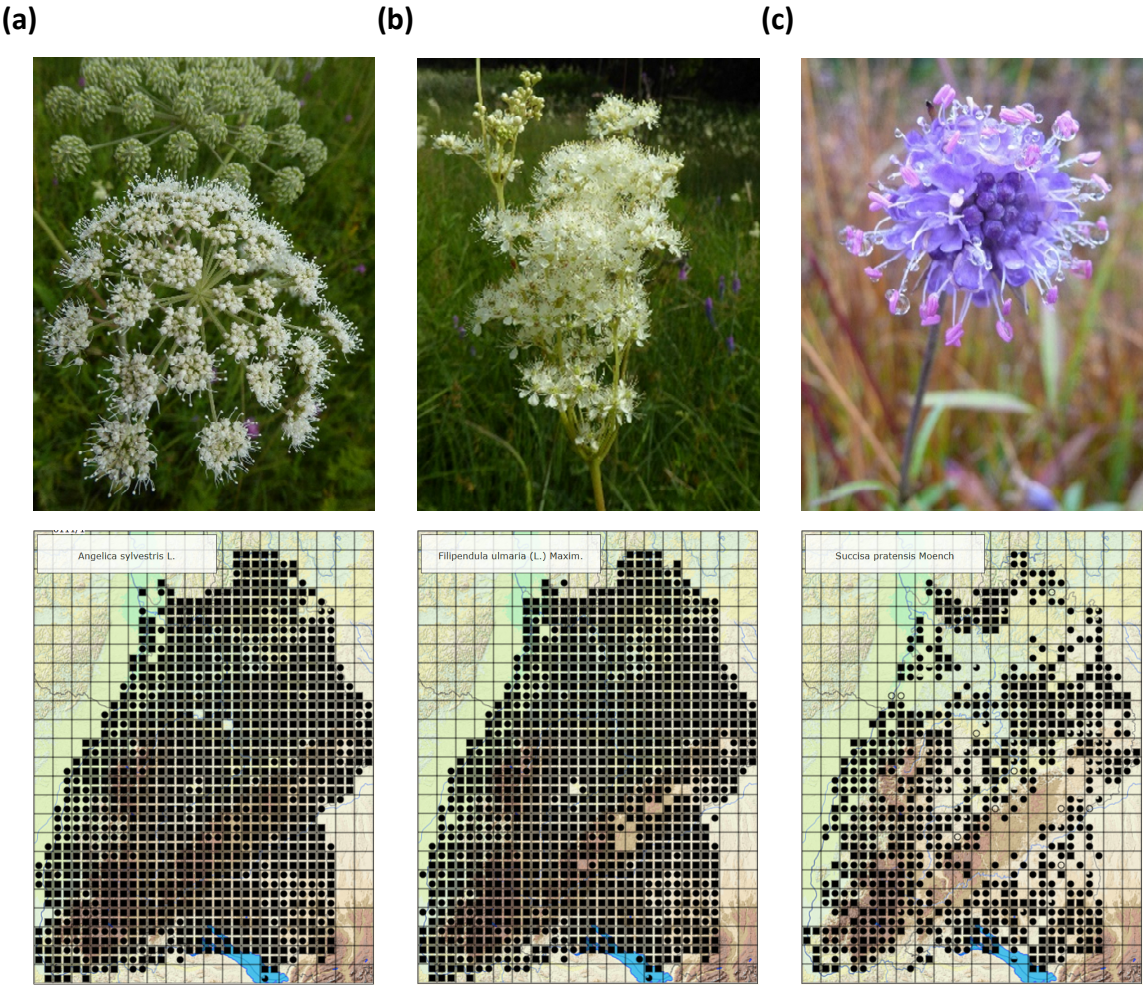


Figure 3.1: *A. sylvestris* (a), *F. ulmaria* (b), *S. pratensis* (c) and their spatial distribution over Baden-Württemberg. [Source: http://www.florabw.recorder-d.de/ Applied: 29 August 2019]

We selected 20 litter meadows distributed across the Allgäu in south-west Germany to study the effect of various environmental factors on genetic variation (Figure 3.2, Table S3.1). The study region is characterized by a temperate climate with precipitation between 900 and 1600 mm/year and annual temperatures from 5.5 to 7.5 °C.

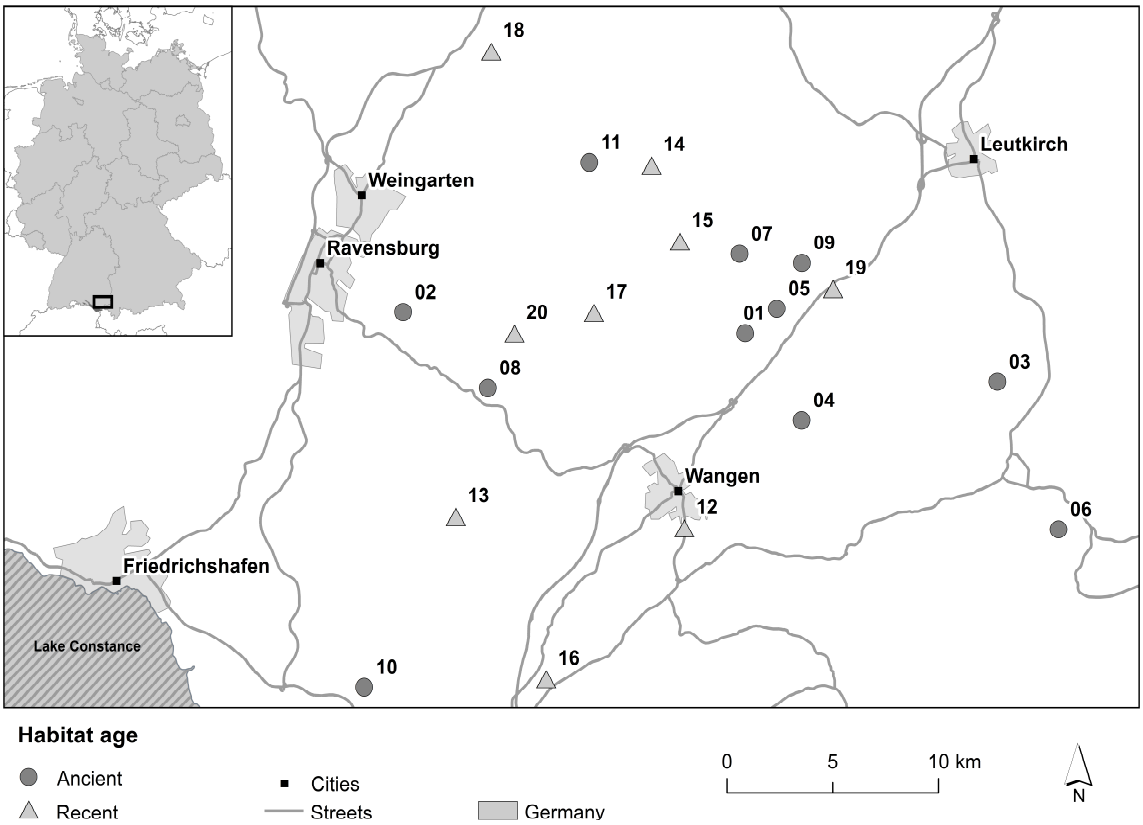


Figure 3.2: Geographic position and habitat age of the analysed populations of *A. sylvestris, F. ulmaria,* and *S. pratensis.*

The land use history of the litter meadows was reconstructed with historical cadastral maps from three different points in time (1800s, 1910/1920s, and 1950s) to investigate the impact of habitat age on genetic variation (Table S3.2). We identified eleven sites as historically old ('ancient'), which have been wet grasslands since before the 1800s, and nine sites as historically young ('recent'), which developed from ponds during the 1900s, applying the software ArcGIS® 10.3.1 (Esri, Redlands, CA, USA).

In a next step, we digitized the oldest cadastral maps available for the area (1800s) as well as current topographical maps (2018) in a 3 km radius around each study site.

Following landscape structures were chosen as potential explanatory variables for genetic diversity (Table S3.3): size of each study site, past and present distance to the nearest settlement, and past and present total area of wet grasslands within each circle. Moreover, we calculated past and present connectivity according to Hanski (1994) as $S_i = \sum_{j \neq I} \exp(-\alpha d_{ij}) A_j$, where S_i is the connectivity of the patch i, d_{ij} is the distance (km) between patches i and j, A_j is the area (ha) of the patch j, and α is the parameter of the exponential distribution setting the influence of distance on connectivity (Helm et al., 2006). Following Lindborg and Eriksson (2004) and Reitalu et al. (2010) α was set to one and not weighted by the dispersal abilities of the plant species in the community.

The cover of vascular plants, mosses, litter, and open soil were incorporated from vegetation surveys to examine the impact of the local habitat quality on genetic diversity (Table S3.4). Furthermore, we aimed to test the influence of the population size on genetic diversity. The population size of each species was, therefore, determined by counting the number of individuals in 10 to 15 1 m^2 plots per study site. The average number of individuals per square metre was then multiplied with the present area size (Reisch et al., 2018). For those study sites, where no individual could be found within the 1 m^2 plots although plant material was collected, the total number of individuals was set from 0 to 1 before multiplying (Table S3.4).

We sampled 16 individuals per population and species for molecular analyses to display more than 90 % of the total genetic diversity (Leipold et al., 2020). The fresh leaf material was frozen in plastic bags in liquid nitrogen and stored at -20 °C until DNA extraction.

Molecular analyses

The DNA extraction was carried out following the CTAB protocol from Rogers and Bendich (1994) modified by Reisch (2007). The DNA quality and concentration were determined with a spectrophotometer. Afterwards, the DNA samples were diluted to the same level of 7.8 ng DNA per µl H_2O. We chose the analysis of amplified fragment length polymorphism (AFLP; Vos et al., 1995) for the analysis of the genetic variation within populations. The AFLP analyses were performed following the standardized protocol of Beckmann Coulter

(Bylebyl et al., 2008; Reisch, 2008). After screening 36 primer combinations per species, three species specific primer combinations were chosen for the selective amplification (Table S3.5). An automated capillary electrophoresis machine (GeXP, Beckmann Coulter) was used to separate the fluorescence-labelled DNA fragments by capillary gel electrophoresis. Virtual gels were analysed manually using the software Bionumerics 4.6 (Applied Maths, Kortrijk, Belgium). Only strong and clearly defined fragments were taken into account for further analyses, while samples without clear banding pattern due to unsuccessful AFLP were repeated or ultimately excluded.

A genotyping error rate was determined to ensure the reproducibility of the AFLP analyses (Bonin et al., 2004). Therefore, 10 % of all investigated samples were analysed twice. The percentage of fragments showing differences between original and replicate lay at 3.61 % (*A. sylvestris*), 5.36 % (*F. ulmaria*), and 4.93 % (*S. pratensis*).

Statistical analyses

The presence or absence of bands per particular fragment and individual was transformed into binary (0/1) matrices in Bionumerics 4.6. Based on these matrices, we calculated the genetic diversity within each population in Popgene 32 (Yeh et al., 1997) as Nei's gene diversity (GD) $H = 1 - \sum(p_i)^2$, with p_i representing the allele frequency.

A Kruskal-Wallis test with a post-hoc-Dunn's test (Dinno, 2015) and following Bonferroni p-adjustment (Bland & Altman, 1995) was calculated in R to compare Nei's gene diversity on species level (R Core Team, 1978). We further tested the dependence of Nei's gene diversity on habitat age.

Hierarchical analyses of molecular variance (AMOVA) based on pairwise Euclidian distances among samples were calculated using the software GenAlEx 6.41 (Peakall & Smouse, 2006). Hence, we analysed the genetic variation within and among populations as well as among populations on ancient and recent sites.

We computed Mantel tests with 999 permutations (Mantel, 1967) to display correlations of geographic and genetic distances (Φ_{PT} values calculated in the AMOVA) among populations.

Wilcoxon-Mann-Whitney tests displayed possible differences between past and present landscape variables (Table S3.6). Correlation tests (Pearson correlation coefficients) were conducted to test for intercorrelations among explanatory variables (ii – xiii) (Table S3.7).

We formulated linear regression models for each species in R (R Core Team 1978) describing the variation of Nei's gene diversity in association to the scaled and centred explanatory variables: (i) habitat age (not scaled and centred), (ii) area size, (iii) past and (iv) present total area of wet meadows, (v) past and (vi) present distance to nearest settlement, and (vii) past and (viii) present connectivity, which were described above. Further data about the coverage of (ix) vascular plants, (x) mosses, (xi) litter, (xii) open soil, and (xiii) population size were included in these models. We ranked all possible linear models according to AICc values (Akaike Information Criterion corrected for small sample sizes) to detect the models with the highest information content (Burnham & Anderson, 2002).

Results

Genetic diversity and differentiation

All studied species revealed similar levels of genetic diversity (Figure 3.3). The mean genetic diversity of *A. sylvestris* populations lay at 0.216, ranging between 0.193 and 0.244. Similar values were found for *F. ulmaria*, whose mean genetic diversity was 0.216, with a minimum of 0.184 and a maximum of 0.248. Mean genetic diversity of *S. pratensis* was slightly lower with 0.210, varying from 0.167 to 0.242 (Table 3.1).

Overall genetic differentiation among populations was low. The differentiation found among populations was estimated at 4 % for *A. sylvestris* and at 5 % for *S. pratense*. *F. ulmaria* showed the highest differentiation rate with 8 % (Table 3.2). However, the AMOVAs showed no genetic differentiation among populations on ancient and recent sites.

Mantel tests revealed no significant correlation between genetic and geographic distances in either species (*A. sylvestris*: r = 0.0527, p = 0.052; *F. ulmaria*: r = 0.0003, p = 0.423; *S. pratense*: r = 0.0026, p = 0.334). Therefore, the studied populations are not likely to be isolated by distance.

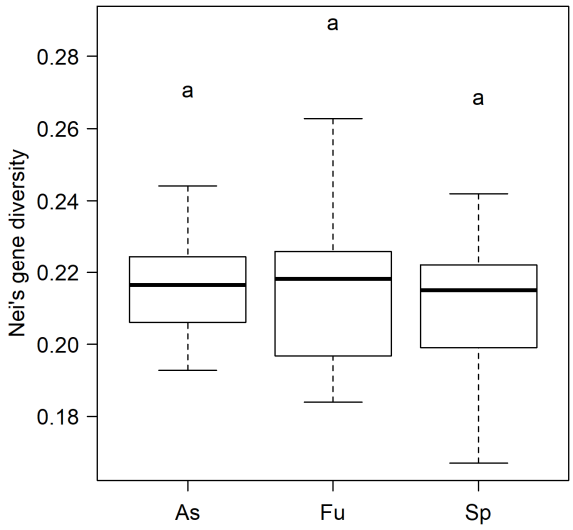


Figure 3.3: Nei's gene diversity of *A. sylvestris* (As), *F. ulmaria* (Fu), and *S. pratensis* (Sp). The results of the Kruskal-Wallis test are indicated by the letters above the boxplot.

Table 3.1: Number (No.), name (Population), and habitat age (Age) of the analysed populations. Also specified is the number of investigated individuals (N) and Nei's gene diversity per population of *A. sylvestris* (As), *F. ulmaria* (Fu), and *S. pratensis* (Sp).

			N			Nei's gene diversity		
No.	**Population**	**Age**	**As**	**Fu**	**Sp**	**As**	**Fu**	**Sp**
01	Arrisried	ancient	16	16	16	0.218	0.248	0.215
02	Schlier	ancient	16	16	16	0.203	0.187	0.205
03	Schwanden	ancient	-	16	16	-	0.220	0.215
04	Ratzenried	ancient	16	15	16	0.216	0.220	0.242
05	Liebenried	ancient	16	16	16	0.226	0.205	0.209
06	Argen	ancient	16	16	16	0.212	0.193	0.188
07	Kißlegg	ancient	15	16	16	0.203	0.209	0.231
08	Rotheidlen	ancient	15	16	16	0.244	0.195	0.207
09	Bremberg	ancient	16	16	16	0.229	0.227	0.218
10	Nitzenweiler	ancient	16	16	16	0.193	0.198	0.179
11	Wolfegg	ancient	16	16	-	0.233	0.236	-
12	Wangen im Allgäu	recent	16	16	16	0.198	0.221	0.199
13	Hinteressach	recent	16	16	16	0.217	0.263	0.220
14	Wolfegg	recent	16	16	16	0.217	0.225	0.167
15	Rotenbach	recent	15	16	16	0.207	0.246	0.230
16	Hüttenweiler	recent	16	16	16	0.206	0.184	0.231
17	Vogt	recent	16	16	16	0.223	0.213	0.222
18	Gwigg	recent	16	16	16	0.213	0.216	0.194
19	Sigrazhofen	recent	16	16	-	0.223	0.190	-
20	Edensbach	recent	16	16	-	0.233	0.222	-
Mean						**0.216**	**0.216**	**0.210**
SE						± 0.003	± 0.005	± 0.004

Table 3.2: Genetic variation per species among populations on ancient and recent sites (habitat age), among and within studied populations detected by AMOVA. Levels of significance are based on 999 iteration steps.

Species	AMOVA	df	SS	MS	Est. Var.	%	Φ_{PT}	
A. sylvestris	Among habitat age	1	19.63	19.63	0.00	0	0.040	***
	Among populations	17	463.21	27.25	0.71	4		
	Within populations	282	4514.20	16.01	16.01	96		
F. ulmaria	Among habitat age	1	53.73	53.73	0.04	0	0.077	***
	Among populations	18	866.09	48.12	1.71	8		
	Within populations	299	6242.00	20.88	20.88	92		
S. pratensis	Among habitat age	1	26.27	26.27	0.00	0	0.053	***
	Among populations	15	393.22	26.21	0.77	5		
	Within populations	255	3539.81	13.88	13.88	95		

Signif. code: $p \leq 0.001$ ***

df, degree of freedom; SS, sum of squares; MS, mean squares; Est. Var., estimated variation; %, proportion of genetic variation; Φ_{PT}, indicator for genetic differentiation among populations

Linear regression models

The AICc model selection generated significant models for all studied species (Table 3.3 a - c). The model for *A. sylvestris* only included a negative association with the present area size, indicating a decrease of genetic diversity with increasing meadow size (Table 3.3 a). Genetic diversity in *S. pratensis* was negatively affected by population size (Table 3.3 c), explaining 21.51 % of the observed variation. For *F. ulmaria* the model revealed more than one connection with the explanatory variables included (Table 3.3 b). Present connectivity was the most important variable negatively influencing current genetic diversity, while past connectivity was positively associated. Present distance to the nearest settlement and present total area of wet meadows were positively related to genetic diversity in this species. Habitat age was also a significant predictor for genetic diversity indicating a tendency for recent meadows to show higher genetic diversity levels. Both moss and vascular plant cover were positively associated with genetic diversity of *F. ulmaria*. Overall, the model accounted for 75.37 % of the observed variation.

Table 3.3: Linear models explaining genetic diversity patterns in *A. sylvestris* (a), *F. ulmaria* (b), and *S. pratensis* (c) populations in litter meadows. The effect size of the association with the response variable (Estimate), the standard error, and the p-value are given for each of the variables within the models.

(a) *A. sylvestris*

		Estimate	**Std. Error**	**p-value**	
	(Intercept)	0.216	0.003	< 0.001	***
Response variable	**Explanatory variable**				
Nei's gene diversity	AREA_S	- 0.007	0.003	0.019	*

Residual standard error: 0.01155 on 17 degrees of freedom
Multiple R-squared: 0.283, Adjusted R-squared: 0.2408
F-statistic: 6.71 on 1 and 17 DF, p-value: 0.01905

(b) *F. ulmaria*

		Estimate	**Std. Error**	**p-value**	
	(Intercept)	0.208	0.003	< 0.001	***
Response variable	**Explanatory variable**				
Nei's gene diversity	Age_recent	0.019	0.005	0.004	**
	AREA_2018	0.023	0.005	< 0.001	***
	CON_2018	- 0.029	0.005	< 0.001	***
	DIST_2018	0.011	0.003	0.002	**
	CON_1800	0.010	0.003	0.005	**
	MOSS	0.010	0.003	0.009	**
	VASC	0.006	0.003	0.042	*

Residual standard error: 0.01074 on 12 degrees of freedom
Multiple R-squared: 0.8444, Adjusted R-squared: 0.7537
F-statistic: 9.304 on 7 and 12 DF, p-value: 0.0004949

(c) *S. pratensis*

		Estimate	**Std. Error**	**p-value**	
	(Intercept)	0.210	0.004	< 0.001	***
Response variable	**Explanatory variable**				
Nei's gene diversity	Population size	- 0.010	0.004	0.035	*

Residual standard error: 0.01768 on 15 degrees of freedom
Multiple R-squared: 0.2642, Adjusted R-squared: 0.2151
F-statistic: 5.385 on 1 and 15 DF, p-value: 0.03481

Signif. codes: $p \leq 0.001$ ***; $0.001 < p \leq 0.01$ **; $0.01 < p \leq 0.05$ *

AREA_S, area size; AREA_2018, present total area of wet meadows [ha]; DIST_2018, present distances to the nearest settlement [km]; CON_1800/CON_2018, past and present connectivity; MOSS, moss cover [%]; VASC, vascular plant cover [%]

Discussion

Genetic diversity and differentiation

We observed similar values of genetic variation within and among populations of all study species. The genetic diversity of these species slightly exceeded the values expected for insect pollinated species (Reisch & Bernhardt-Römermann, 2014). Genetic differentiation among populations was generally low, with *F. ulmaria* showing the highest differentiation. Spatial isolation did not play a major role for population differentiation.

Previous studies have shown that seeds are well transported among meadows via mowing machines (Strykstra et al., 1997). The litter meadows investigated here are typically mown by only few conservation managers once in the autumn (personal communication), enhancing gene flow by seed exchange among sites. Additionally, the occurrence of the study species is not strictly limited to litter meadows (Oberdorfer et al., 2001) and they are pollinated by a diverse group of insects (Kühn et al., 2004), providing many opportunities for gene flow by pollinators among sites.

Other studies on genetic diversity and differentiation of the species analysed here are scarce. Only the effect of inbreeding and population size on the genetic variation of *S. pratensis* was already studied using allozyme electrophoresis (Vergeer et al., 2003). Therefore, the genetic variation observed in these species is not directly comparable with other studies.

Effects of habitat age on genetic variation

Levels of genetic diversity in all three study species were similar among populations on ancient and recent sites. Additionally, habitat age revealed no significant impact on genetic diversity in *A. sylvestris* and *S. pratensis* in the linear regression models. This result stands in contrast to the studies of Jacquemyn et al. (2004) and Rosengren et al. (2013), who observed a comparatively lower genetic diversity on recent sites, e.g. in the moss species *Homalothecium lutescens* (Hedw.) H. Rob. However, historic management practices of sowing, hay and seedling transfer for the establishment and maintenance of litter meadows (Poschlod, 2017; Poschlod & Fischer, 2016) likely supported high levels of gene flow between ancient and recent sites. Moreover, all study species are pollinated by numerous

different insects (Kühn et al., 2004) increasing the levels of gene flow among sites. Thus, gene flow by pollinators and seed dispersal at the time of founding and afterwards might reduce the effects of habitat age (Vandepitte et al., 2010).

Habitat age was a significant predictor for genetic diversity patterns of *F. ulmaria*, revealing a tendency of more recent sites to show higher diversity values. However, the variable 'habitat age' was possibly included by the model selection algorithm to correct for the overestimation of 'past connectivity', which is significantly lower today. Therefore, we conclude that habitat age generally had no impact on genetic diversity of our study species.

Furthermore, we observed no significant differentiation among populations concerning habitat age. The practice of litter meadow establishment and traditional management practices ensured high levels of gene flow in the past. Today, seeds are still comparatively well transported via mowing machines among litter meadows (Strykstra et al., 1997). These land use practices supported and still support relatively high levels of gene flow preventing genetic differentiation among populations on ancient and recent sites.

Effects of landscape structure on genetic diversity

Genetic diversity in *A. sylvestris* was negatively associated with the area of the respective litter meadow indicating larger meadows to comprise lower genetic diversity. Larger habitats are expected to sustain larger populations and thus, also higher genetic diversity (Ouborg et al., 2006). In the case of *A. sylvestris* neither genetic diversity nor habitat size correlated with population size. *A. sylvestris* might colonize microsites instead of whole meadows due to variable local habitat conditions and is also not limited to litter meadows as habitat, which might falsify the impact of population size. Furthermore, habitat size was determined via topographic maps leading to a potential over- or underestimation of litter meadows' habitat size. Therefore, we assume no or only a weak impact of habitat size on the genetic diversity of *A. sylvestris* populations.

Past and present landscape structures revealed the greatest impact on the genetic diversity of *F. ulmaria* populations. The total present area of wet meadows, the present distance to the next settlement, and the past and present connectivity were associated with genetic diversity levels. All these factors have previously been shown to influence

genetic diversity in grassland species (Jacquemyn et al., 2004; Münzbergová et al., 2013; Reitalu et al., 2010).

Genetic diversity in *F. ulmaria* increased with the present area of wet meadows around the studied populations. A large patch size and a high proportion of habitats within a geographic region is frequently found to increase genetic diversity by improving patch connectivity via pollinators or other gene flow vectors (Ouborg et al., 2006; Prentice et al., 2006). Gene flow among closely located patches decreases the effects of inbreeding and genetic drift and thus, maintains high genetic diversity (Aguilar et al., 2008).

The present distance to the nearest settlement revealed a positive impact on the genetic diversity of *F. ulmaria*. It is generally accepted that anthropogenic disturbance levels decrease with increasing distance to the next settlement. Since comparatively low levels of man-made disturbance led to an increase of both species and genetic diversity (Frey et al., 2016), genetic diversity levels in *F. ulmaria* increased with rising distance to the nearest settlement.

We found a positive impact of past connectivity on the genetic diversity in *F. ulmaria* complying with the findings of Münzbergová et al. (2013), who observed a positive effect of historic habitat connectivity on genetic diversity of *S. pratensis*. In the past, traditional management of litter meadows included frequent sowing or transplanting of plant material to increase the vegetation cover of desired litter producing species (Poschlod, 2017). These management practices, which may have positively affected undesired species as well, maintained high gene flow levels across the whole region. High connectivity among sites may increase colonization and reduce extinction rates, explaining the positive effect of past connectivity on the genetic diversity in *F. ulmaria*.

However, present connectivity revealed an opposite effect on the genetic diversity in *F. ulmaria*. The cultivation of litter meadows became redundant during the last decades and thus, remaining species-rich litter meadows within the study region are managed by only few conservation managers today (personal communication). Moreover, seeds of all study species are fully developed during mowing season in late autumn (Poschlod et al., 2003) and are likely to be transported well via mowing machines (Strykstra et al., 1997) creating ‘too much’ gene flow among populations. Exceptionally high levels of gene flow

may induce an impoverishment of the local gene pool due to genetic 'swamping' and thus, cause a negative impact of present habitat connectivity on genetic diversity in *F. ulmaria*.

Effects of habitat quality and population size on genetic diversity

The genetic diversity in *F. ulmaria* was positively associated with moss and vascular plant cover. In a vegetation unit, the frequent abundance of mosses and vascular plants is expected to decrease germination and establishment of plant species (Drake et al., 2018; Poschlod & Biewer, 2005; Špačková et al., 1998). However, in wet grassland habitats mosses can act as safe sites for germination (Wang et al., 2012) by retaining seeds (Freestone, 2006), producing more stable habitat conditions, and protecting seedlings from harsh climatic conditions (Donath & Eckstein, 2010; Lemke et al., 2015). Similarly, grass tussocks can also retain seeds and facilitate germination, especially in wet environments (Wang et al., 2012). A high cover of mosses and vascular plants may, therefore, facilitate the germination and establishment of *F. ulmaria* in litter meadows and consequently increase genetic diversity levels.

Correlations between population size and genetic diversity are expected to be positive, with larger populations maintaining more genotypes (Ouborg et al., 2006; Vergeer et al., 2003). However, the genetic diversity of *S. pratensis* decreased with increasing population size. Grassland plant species with long life cycles, slow intrinsic dynamics, and comparatively large population size may occur as remnant populations in modern landscapes (Maurer et al., 2003). Piqueray et al. (2011) observed, moreover, that historic habitat configurations may often affect the present occurrence of a species, indicating a time lag between habitat loss, fragmentation, and their consequences on genetic diversity (Helm et al., 2006). Therefore, previous studies predicted a delayed response of genetic diversity to habitat fragmentation (Honnay et al., 2007). Additionally, *S. pratensis* is a more specialised and less widespread species than *A. sylvestris* and *F. ulmaria*. The Pearson correlation revealed a negative impact of moss cover on the population size of *S. pratensis* and, moreover, a negative relationship between the cover of moss and open soil. Therefore, we hypothesize that *S. pratensis* depends on open soil for successful germination and establishment. Hence, genetic diversity levels were low, despite high

population size, due to a potential extinction debt and/or missing niches for germination and establishment.

Conclusions

Our study revealed significant and species specific impacts of landscape structure, habitat quality, and population size on genetic diversity. While the influence of habitat size on genetic diversity in *A. sylv*estris remained unclear, *F. ulmaria* populations were significantly driven by the distance to the nearest settlement, the total area of litter meadows, and their connectivity. Moreover, the cover of mosses and vascular plants showed a significant impact on the genetic diversity of *F. ulmaria* populations. The genetic diversity of *S. pratensis* populations was affected in two ways: directly by population size and indirectly by the cover of mosses.

Abandonment of traditional land use practices changed the abundance and local habitat quality of semi-natural litter meadows during the last decades. Additionally, the practice of litter meadow establishment, traditional and also current management practices, caused and still cause man-made gene flow among litter meadows. Thus, past and present landscape structures as well as local habitat quality turned out as key variables driving genetic variation patterns of typical litter meadow species.

Hence, the future conservation of these species rich habitats should pay reference to past as well as present processes to ensure the maintenance of litter meadows in our cultural landscape. Different mowing machines should be used in a rotating system to ensure moderate levels of gene flow and thus, counteract an impoverishment of the gene pool by genetic ‘swamping’. Furthermore, traditional management practices should be supported to promote appropriate germination niches.

CHAPTER 4

THE IMPACT OF ENVIRONMENT ON GENETIC AND EPIGENETIC VARIATION OF *TRIFOLIUM PRATENSE* L. POPULATIONS IN TWO CONTRASTING CENTRAL EUROPEAN GRASSLANDS

Theresa Anna Lehmair, Peter Poschlod, and Christoph Reisch

Trifolium pratense L.

Abstract

Central European grasslands are characterized by diverse environmental conditions and management regimes. Examples are nutrient poor, extensively managed calcareous grasslands or comparatively nutrient-rich, intensively used oat-grass meadows. The aim of our study was to test whether populations from these two contrasting habitats differ in genetic or epigenetic variation and to identify drivers of genetic and epigenetic variation.

We analysed the genetic and epigenetic variation of the ecologically variable plant species *Trifolium pratense* using amplified fragment length polymorphism (AFLP) and methylation-sensitive amplification polymorphism (MSAP) analyses.

Levels of genetic and epigenetic differentiation were low between contrasting habitats and among populations. Genetic distances correlated significantly with habitat dissimilarity, but neither genetic nor epigenetic variation revealed isolation by distance. Genetic and epigenetic diversity were not interdependent and did not show significant differences among calcareous grassland and oat-grass meadow populations. However, we observed a significant correlation of epigenetic diversity with soil moisture and soil pH, while genetic diversity was not affected by environment.

Our results demonstrated that genetic and also epigenetic variation may depend on different environmental conditions. Genetic variation was affected more strongly by habitat specific environmental conditions induced by land use related disturbance and gene flow patterns. Epigenetic variation was driven by challenging environmental conditions and decreased, therefore, under drought and high pH, with the latter potentially resulting in phosphorus limitation.

Key words

epigenetic variation; genetic variation; environmental conditions; *Trifolium pratense*

Introduction

Hutchinson (1957) defined the concept of 'habitat' as a collection of environmental conditions allowing a plant species to survive and to grow. Applying this definition, each habitat represents a specific environmental setting with certain selective pressures (Wu et al., 2013). Plant species need to respond to specific soil or climatic conditions to cope with these pressures. Furthermore, they are subjected to different management regimes in anthropogenic habitats, such as semi-natural grasslands. Type, intensity, and time of management may cause large differences in the plant composition of Central European grasslands. Mowing, for instance, happens abruptly and affects all species simultaneously, while more continuously grazing never pertains a population on the whole (Reisch & Poschlod, 2009). Widespread and common species such as *Trifolium pratense* often have a very broad ecological niche and may occur in grassland types of different ecological conditions and management.

Previous studies assumed that environmental conditions may affect the genetic code of a plant species indirectly (Billeter et al., 2002; Hooftman et al., 2004; Vandepitte et al., 2007) indicating that the reaction of a plant species to changing environmental conditions is exclusively based on genetic variation (Wu et al., 2013). During the last decades, numerous studies demonstrated that plant species can react to diverse environments without changing their DNA sequence (e.g. Lira-Medeiros *et al.* 2010; Paun *et al.* 2010; Herrera & Bazaga 2011; Schulz, Eckstein & Durka 2013, 2014; Wu *et al.* 2013). These metastable, but heritable changes in gene expression are induced by chemical DNA and histone modifications as well as interference by small non-coding RNAs (Schulz et al., 2014).

The potential reversible DNA methylation of cytosine represents the most studied epigenetic mechanism with important effects on ecologically relevant traits (Foust et al., 2016; Herrera & Bazaga, 2008). Cytosine methylations occur throughout the genome in all sequence contexts (Law & Jacobsen, 2010) and are predominantly located in repetitive sequences and transposable elements (Schulz et al., 2013). From there, cytosine methylations could regulate transposon silencing and gene expression without changing the underlying genetic code (Lele et al., 2018). Methylation-sensitive amplification

polymorphism (MSAP) analyses, established by Schulz *et al.* (2013), allow the identification of methylation-based epiallelic markers in natural populations of non-model plants (Herrera & Bazaga, 2010). These markers enable a genome-wide snapshot of epigenetic variation. Nevertheless, information about their function in natural populations is still scarce (Foust et al., 2016), since only few studies addressed the impact of epigenetic variation on genetically diverse, non-model plant species so far (Abratowska, Wasowicz, Bednarek, Telka, & Wierzbicka, 2012; Herrera & Bazaga, 2010; Lira-Medeiros et al., 2010; Wu et al., 2013).

Changes in DNA methylation were observed to increase in response to biotic and abiotic stressors (Dowen et al., 2012; Herrera, Pozo, & Bazaga, 2012; Verhoeven, Jansen, van Dijk, & Biere, 2010) such as herbivores (Herrera & Bazaga, 2013), salinity (Foust et al., 2016), drought (Labra et al., 2002), extreme temperatures, or nutrient limitation (Boyko et al., 2010). DNA methylation alterations, caused by challenging environmental conditions, are common, sequence-independent, readily generated, and mostly heritable (Verhoeven et al., 2010). Thus, epigenetic variation, provoked by DNA methylation, provides a valuable tool for plant species to rapidly adapt and survive under challenging environmental conditions (Bossdorf et al., 2008). Hereby, different challenging environmental conditions may induce hypo- or hypermethylation or shifts in global methylation patterns depending on plant species or rather genotype (Labra et al., 2002; Schulz et al., 2014; Verhoeven et al., 2010).

During the last decades, numerous studies on various plant species observed profound effects of environmental conditions on both genetic and epigenetic variation patterns (e.g. Billeter et al., 2002; Herrera & Bazaga, 2011; Hooftman et al., 2004; Lira-Medeiros et al., 2010; Richards et al., 2010; Vandepitte et al., 2007). Thus, most plant species are diverse as a result of complex interactions between genetic, epigenetic, and environmental variation (Richards et al., 2010). Previous studies stated a certain correlation of genetic and epigenetic variation (Abratowska et al., 2012; Lira-Medeiros et al., 2010). Hence, epigenetic variation may be controlled by the underlying genetic code (Richards, 2006), but environmental parameters can also directly change epigenetic variation (Jablonka & Raz, 2009). In the studies mentioned above, epigenetic differentiation was,

therefore, generally more closely related to environment than to genetic differentiation. Thus, they indicate that heritable epigenetic changes might constitute a key variable for local adaptation (Richards et al., 2010). Therefore, genetic and epigenetic variation should be tested for interdependence when considering environmental impact factors on genetic and epigenetic variation.

We asked the following questions to gain a better understanding about the impact of contrasting environmental conditions on genetic and epigenetic variation in *T. pratense*: (i) Are populations genetically and/or epigenetically differentiated among contrasting grassland habitats or are they isolated by distance? (ii) Does genetic and/or epigenetic diversity differ between calcareous grassland and oat-grass meadow populations? (iii) What is the impact of environment on genetic and/or epigenetic diversity levels? (iv) Is genetic and epigenetic variation of *T. pratense* populations interdependent?

Methods

Study design

For our study, we selected calcareous grasslands and oat-grass meadows, five each, all over the Swabian Alb in south-west Germany (Figure 4.1, Table S4.1). Semi-natural calcareous grasslands on the Swabian Alb are characterized by steep slopes, shallow soils, and relative dry soil conditions (Wilmanns, 1955). They are mainly grazed by sheep from late spring until early summer (Reisch & Poschlod, 2009). Continuous, selective grazing and physical disturbance by trampling impoverish soil nutrients and shape the heterogeneous soil and sward structure of this habitat type (Olff & Ritchie, 1998). Oat-grass meadows are traditionally managed with two (or three) cuttings per year. Manure and more recently mineral fertilizer are applied to maintain productivity (Poschlod, 2017; Poschlod et al., 2009). These lowland hay meadows show a more unified soil and sward structure than calcareous grasslands, since mowing affects all species simultaneously and in the same way (Ellenberg, 1996). Both habitats reveal contrasting environmental conditions although they are located nearby each other within the same geographic region. Therefore, calcareous grasslands and oat-grass meadows of this region appeared as promising model system for studying genetic and epigenetic variation patterns.

The widespread species *Trifolium pratense* L. (Figure 4.2) occurs in calcareous grasslands and oat-grass meadows. Therefore, it represents an appropriate model organism to analyse genetic and epigenetic variation within these contrasting habitats. The red clover (Fabaceae, 2n=14) is flowering between June and September (Kühn et al., 2004). It is nearly exclusively pollinated by bumble bees and the persistent seeds may survive at least 39 years within the soil seed bank (Toole & Brown, 1946). *T. pratense* is an essential species for profitable grassland management due to its high fodder value (Dierschke & Briemle, 2002) and its ability to improve soil properties by nitrogen fixation (Carlsson & Huss-Danell, 2003).

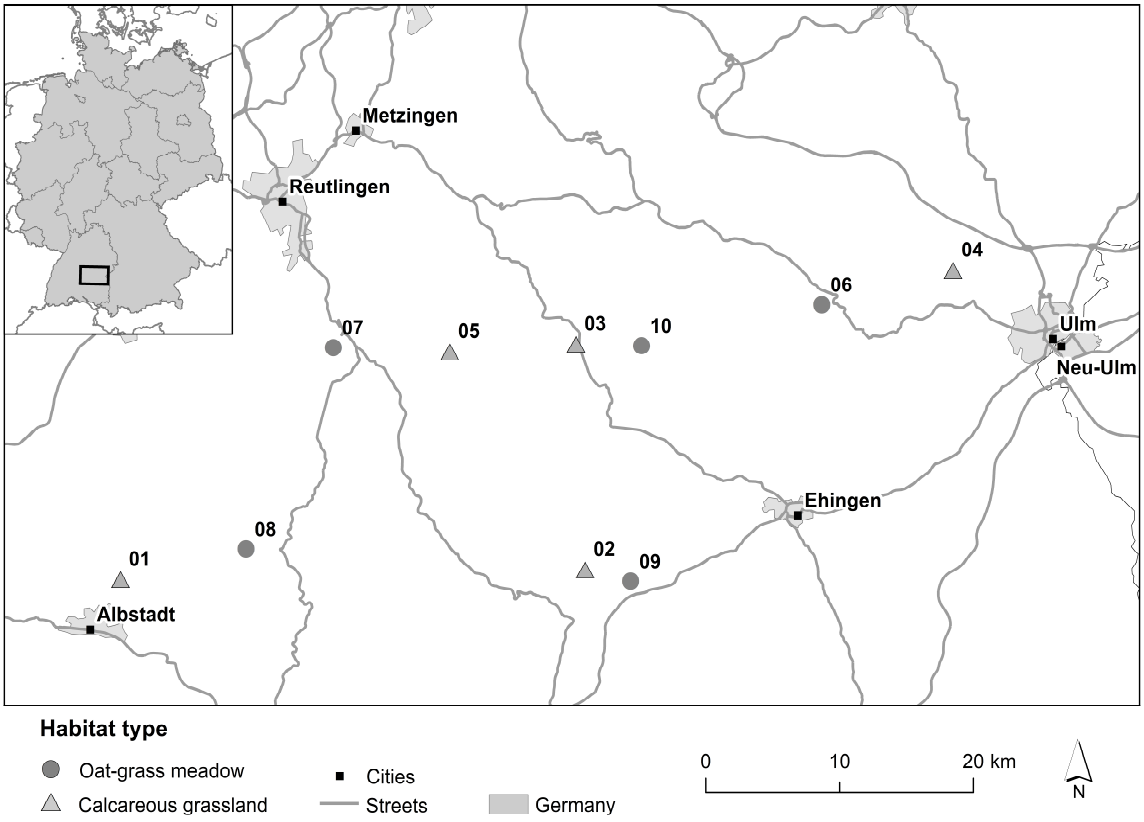


Figure 4.1: Geographic position of the analysed populations on calcareous grasslands (triangles) and oat-grass meadows (points), five each.

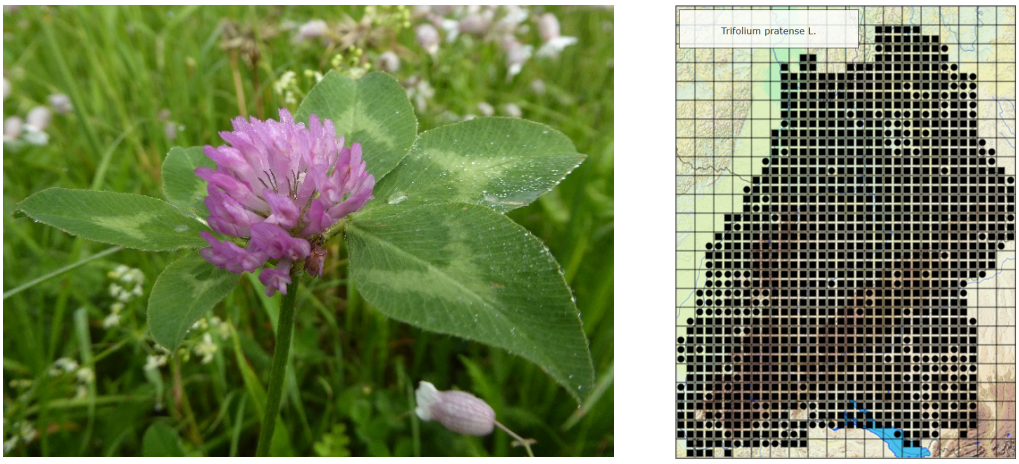


Figure 4.2: *T. pratense* and its spatial distribution over Baden-Württemberg. [Source: http://www.florabw.recorder-d.de/ Applied: 29 August 2019]

Ellenberg indicator values, using plants as bio-indicators, were applied to gain information about environmental conditions. Environmental conditions may often fluctuate in time and space and can, thus, not be estimated in a single measurement (Diekmann, 2003). The indicator values have advantages over conducting measurements (Zonneveld, 1983), since plants represent the integrated expression of the values of those environmental variables. Furthermore, measurements rely on technical equipment and often need more time and financial effort than floristic observations. Ellenberg indicator values, established by Ellenberg et al. (1992), represent the realized optima of a species. They are expressed as ordinal numbers reflecting the species' requirements along e.g. light, soil moisture, soil reaction/pH, soil nutrients, soil salinity, or temperature gradients. The availability of light, nutrients as well as soil moisture and pH represent the local environmental conditions of a habitat (Ellenberg, 1996). Therefore, we calculated the mean weighed light, soil moisture, soil reaction/pH, and soil nitrogen Ellenberg indicator values per study site using the species' abundance from previously conducted vegetation surveys (unpublished data) as described by Diekmann (2003). These indicator values will be named simplified as light, soil moisture, soil pH, and soil nutrients throughout this study.

For molecular analyses we took leaf samples from 16 individuals per population and species to cover more than 90 % of the total (epi)genetic diversity (Leipold et al., 2020).

Genetic and epigenetic fingerprinting

All 160 individuals were analysed genetically and epigenetically. DNA was extracted following the CTAB protocol from Rogers and Bendich (1994) modified by Reisch (2007). A spectrophotometer was used to measure DNA quality and concentration. All DNA samples were diluted to the same level of 7.8 ng DNA per µl H_2O.

Genetic variation within populations was determined using genome-wide genotyping with amplified fragment length polymorphisms (AFLP; Vos *et al.* 1995). The AFLP analyses were performed following the standardized protocol of Beckmann Coulter (Bylebyl et al., 2008; Reisch, 2008). After a screening of 42 primer combinations we selected three appropriate combinations for the selective amplification (Table S4.2).

Methylation-sensitive amplified polymorphism (MSAP) analyses were performed in accordance to the technique of Schulz, Eckstein and Durka (2013). Thus, MSAP analyses follow the protocol of modified AFLP analyses replacing the frequent cutter *MSeI* by two isoschizomers *HpaII* and *MspI*. These restriction enzymes attach at the same tetranucleotide (5'CCGG) sequence with differing sensitivity to cytosine methylation states and cover, thus, the most frequent methylation types in the CG and CHG (with H = A, C or T) sequence context (Law & Jacobsen, 2010; Schulz et al., 2013). Therefore, they allow the comparison of large amounts of anonymous, methylation sensitive CCGG regions across the genome for a large number of individuals (Schulz et al., 2013). 36 primer combinations were screened to identify three suitable combinations for the selective amplification (Table S4.2).

The fluorescence-labelled DNA fragments were separated by capillary gel electrophoresis using an automated capillary electrophoresis machine (GeXP, Beckmann Coulter). Samples without clear banding pattern were repeated and only strong and clearly defined fragments were taken into account for further analyses. Fragment data were analysed manually with the software Bionumerics 7.6.2 (Applied Maths, Kortrijk, Belgium).

After fragment detection we applied the 'mixed scoring 2' by Schulz, Eckstein and Durka (2013) to score the presence-absence matrices for MSAP fragments. Schulz *et al.* (2013) defined four conditions for the resulting *EcoRI*/*HpaII* and *EcoRI*/*MspI* fragment profiles: (i) fragments are present in both profiles (unmethylated state/u-type), (ii) fragments are present only in *EcoRI*/*MspI* profiles (hemi- or fully methylated at the internal cytosine/m-type), (iii) fragments are present only in *EcoRI*/*HpaII* profiles (hemi-methylated at the external cytosine/h-type), and (iv) complete absence of fragments in both profiles (uninformative state).

The reproducibility of the AFLP and MSAP analyses was tested by calculating the genotyping error rate (Bonin et al., 2004). 10 % of all analysed samples were replicated twice and the percentage of fragments with differences between original and replicate was evaluated. The genotyping error rates for AFLP analyses were 5.24 % and for MSAP analyses 1.02 %.

Data analyses

Genetic and epigenetic differentiation within and among populations as well as between habitat types were partitioned with hierarchical analyses of molecular variance (AMOVA). AMOVAs were calculated based on pairwise Euclidian distances among samples using the software GenAlEx 6.41 (Peakall & Smouse, 2006).

A correlation between genetic and epigenetic distance matrices was examined applying a simple Mantel test. Geographic and habitat dissimilarity matrices were also checked for correlation patterns. Genetic and epigenetic IBD (isolation by distance) and IBH (isolation by habitat dissimilarity) were tested performing simple and partial Mantel tests with 9,999 permutations applying the 'vegan' library in R (Oksanen et al., 2019). Epigenetic and genetic distance matrices were calculated within the AMOVA (Φ_{PT} values; Table S4.3). Geographic distances [km] were calculated from coordinates and for habitat types, a habitat dissimilarity matrix was constructed by coding pairs of calcareous grassland/oat-grass meadow populations by '1' and pairs of equal habitats by '0' (Table S4.4).

Although simple and partial Mantel tests are suitable to test dissimilarity hypotheses (Legendre et al., 2015; Legendre & Fortin, 2010), e.g. for IBD, they were criticized to show inflated type I error and low statistical power (Diniz-Filho et al., 2013; Guillot & Rousset, 2013; Legendre et al., 2015). Since the controversy on their validity in hypothesis testing remains unresolved (Herrera, Medrano, & Bazaga, 2017), Wang's (2013) method based on multiple matrix regression with randomization (MMRR) was additionally performed. Instead of correlation analyses with removed effects of geography or habitat dissimilarity, this method simultaneously applies the effects of geographic distance and habitat dissimilarity on genetic or epigenetic distance matrices. Distance matrices were scaled and centred to obtain comparable standardized linear regression coefficients (Herrera et al., 2017) before using the MMRR function of Wang (2013) available from the Dryad Data Repository (doi:10.5061/dryad.kt71r).

Genetic and epigenetic diversity within populations were determined using the R Script 'MSAP_calc' (Schulz et al., 2013). Applying the function 'descriptive_parameters', (i) percentage of total and private bands, (ii) percentage of polymorphic loci and subepiloci, and (iii) mean Shannon's information index were calculated with $SI = -\sum p_i \cdot \log_2 p_i$, where p_i

is the frequency of the (epi)genetic marker score ’1’ within the population. The acronyms ‘SI_{gen}’ and ‘SI_{epigen}’ stand for the mean Shannon’s information index and will be substituted by the terms ‘genetic diversity’ and ‘epigenetic diversity’ in the discussion.

Two-sided T-tests (and Wilcoxon-Mann-Whitney tests if necessary) were calculated to examine differences of SI_{gen}, SI_{epigen}, and environmental parameters (light, soil moisture, soil pH, and soil nitrogen) between calcareous grassland and oat-grass meadow populations.

Possible correlation of SI_{gen} and SI_{epigen} with light, soil moisture, soil pH, and soil nitrogen were analysed with correlation tests (Pearson correlation coefficients) applying the ‘PerformanceAnalytics’ (Peterson & Carl, 2019) and ‘Hmisc’ (Harrel Jr & Others, 2019) libraries in R.

Differences between SI_{gen} and SI_{epigen} were examined with paired T-tests. Additionally, SI_{gen} and SI_{epigen} were tested for interdependence applying the correlation tests as mentioned above. Unless otherwise stated the R environment (R Core Team, 1978) was used for statistical analyses.

Results

Genetic and epigenetic differentiation

Hierarchical AMOVA of genetic data (Table 4.1) revealed a global Φ_{PT} of 0.07 with a differentiation between habitat types of 3 % and a differentiation among populations of 4 %. The hierarchical AMOVA of the combined epigenetic data set resulted with 0.05 in a lower Φ_{PT}. 1 % of epigenetic variance resided between habitat types and 4 % among populations. Values of epigenetic differentiation for h-, m-, and u-subepiloci are given in Table 4.1.

A simple Mantel test revealed no correlation between genetic and epigenetic differentiation across all populations ($r = 0.30$; $p = 0.069$). Geographic distance (IBD) and habitat dissimilarity (IBH) were also not correlated ($r = - 0.09$; $p = 0.776$).

Simple and partial mantel tests as well as MMRR revealed no significant relationship between genetic or epigenetic differentiation and geographic distance (IBD) ($p > 0.05$; Table 4.2 & Table 4.3). However, genetic differentiation correlated significantly with habitat dissimilarity (IBH) in simple ($r = 0.51$; $p = 0.004$) and partial ($r = 0.50$; $p = 0.003$) Mantel tests (Table 4.2) as well as MMRR ($r = 0.02$; $p = 0.010$) (Table 4.3). Epigenetic differentiation showed no correlation with habitat dissimilarity (IBH) ($p > 0.05$; Table 4.2 & Table 4.3).

Table 4.1: Genetic variation among populations of different habitat types, among and within studied populations detected by AMOVA.

	AMOVA	df	SS	MS	Est. Var.	%	Φ_{PT}	
AFLP loci	Among habitats	1	46.54	46.54	0.36	3	0.070	***
(n=124)	Among populations	8	140.50	17.56	0.44	4		
	Within populations	150	1584.44	10.56	10.56	93		
MSAP								
all subepiloci	Among habitats	1	109.89	109.89	0.42	1	0.050	***
(n=408)	Among populations	8	608.23	76.03	1.93	4		
	Within populations	150	6767.38	45.12	45.12	95		
h-subepiloci	Among habitats	1	24.20	24.20	0.16	2	0.080	***
(n=116)	Among populations	8	92.50	11.56	0.35	6		
	Within populations	150	885.88	5.91	5.91	92		
m-subepiloci	Among habitats	1	38.63	38.63	0.11	1	0.039	***
(n=144)	Among populations	8	240.40	30.05	0.67	3		
	Within populations	150	2897.25	19.32	19.32	96		
u-subepiloci	Among habitats	1	47.06	47.06	0.16	1	0.051	***
(n=148)	Among populations	8	275.33	34.42	0.91	4		
	Within populations	150	2984.25	19.90	19.90	95		

p values were calculated with 999 iteration steps; Sign. code: $p \leq 0.001$ ***

df, degree of freedom; SS, sum of squares; MS, mean squares; Est. Var., estimated variation; %, the proportion of genetic variation; Φ_{PT}, indicator for genetic differentiation among populations

Table 4.2: Results of simple and partial Mantel tests for genetic and epigenetic pairwise population Φ_{PT} with geographic distance [km] and habitat dissimilarity matrices.

	Geographic distance matrix				Habitat dissimilarity distance matrix			
	Simple test		Partialled on habitat dissimilarity		Simple test		Partialled on geographic distance	
	r	p	r	p	r	p	r	p
AFLP	- 0.08	0.652	- 0.04	0.571	0.51	0.004	0.50	0.003
MSAP								
all subepiloci	- 0.16	0.795	- 0.14	0.767	0.20	0.113	0.19	0.120
h-subepiloci	- 0.11	0.686	- 0.09	0.653	0.22	0.099	0.21	0.108
m-subepiloci	- 0.22	0.896	- 0.21	0.880	0.12	0.237	0.10	0.273
u-subepiloci	- 0.02	0.540	0.00	0.504	0.18	0.142	0.18	0.135

p values were calculated with 9,999 permutations

Table 4.3: Summary of multiple matrix regression with randomization (MMRR) relating genetic and epigenetic distance matrices [Φ_{PT}] with geographic [km] and habitat dissimilarity distance matrices.

			Linear predictor matrices			
	Overall regression		Geographic distance		Habitat dissimilarity	
Differentiation matrix	**F**	**p**	**Coefficient**	**p**	**Coefficient**	**p**
AFLP	10.93	0.014	0.001	0.698	0.015	0.010
MSAP						
all subepiloci	2.10	0.189	- 0.002	0.566	0.004	0.017
h-subepiloci	2.73	0.139	- 0.004	0.599	0.011	0.015
m-subepiloci	1.58	0.299	- 0.004	0.373	0.002	0.143
u-subepiloci	1.27	0.342	0.001	0.845	0.004	0.055

p values were calculated with 9,999 permutations

Genetic and epigenetic diversity

AFLP analyses resulted in 124 fragments. Investigation of genetic diversity across populations revealed mean values of 98.9 % bands per population, no private bands, 49.6 % polymorphic loci, and a mean Shannon's information index (SI_{gen}) of 0.35 (Table 4.4).

A total of 159 MSAP fragments were analysed and scoring revealed 408 markers consisting of 116 h-epiloci, 144 m-epiloci, and 148 h-epiloci. Generally, epigenetic diversity across populations showed mean values of 73.7 % bands per population, 0.8 % private bands, 69.3 % polymorphic subepiloci, and a mean Shannon's information index (SI_{epigen}) of 0.46 (Table 4.4). Further values of epigenetic diversity for h-, m-, and u-subepiloci are given in Table 4.4.

Table 4.4: Measures of the genetic and epigenetic diversity within the analysed populations of *T. pratense*.

	AFLP	**MSAP all**	**MSAP h-subepiloci**	**MSAP m-subepiloci**	**MSAP u-subepiloci**
Number of loci	124	408	116	144	148
Bands per population [%]					
01	99.2	71.8	42.2	84.7	82.4
02	98.4	71.3	39.7	82.6	85.1
03	98.4	69.4	43.1	81.3	78.4
04	100.0	72.5	44.8	78.5	88.5
05	99.2	76.0	52.6	84.0	86.5
06	97.6	74.3	46.6	84.0	86.5
07	97.6	81.6	60.3	88.2	91.9
08	97.6	70.1	33.6	82.6	86.5
09	99.2	75.2	47.4	81.9	90.5
10	99.2	74.5	42.2	83.3	91.2
Mean	**98.6**	**73.7**	**45.3**	**83.1**	**86.8**
SE	± 0.3	± 1.1	± 2.3	± 0.8	± 1.3

Private bands per population [%]					
01	0.0	1.7	6.0	0.0	0.0
02	0.0	0.5	0.9	0.7	0.0
03	0.0	1.0	2.6	0.7	0.0
04	0.0	0.5	1.7	0.0	0.0
05	0.0	0.5	0.9	0.7	0.0
06	0.0	0.0	0.0	0.0	0.0
07	0.0	1.0	2.6	0.7	0.0
08	0.0	1.0	2.6	0.0	0.7
09	0.0	1.2	2.6	0.7	0.7
10	0.0	0.7	2.6	0.0	0.0
Mean	**0.0**	**0.8**	**2.2**	**0.3**	**0.1**
SE	± 0.0	± 0.2	± 0.5	± 0.1	± 0.1
Percentage of polymorphic loci					
01	46.8	68.1	42.2	79.2	77.7
02	52.4	68.1	39.7	79.2	79.7
03	50.0	64.0	43.1	73.6	71.0
04	50.8	67.7	44.8	75.0	78.4
05	48.4	71.8	52.6	78.5	80.4
06	45.2	69.1	46.6	79.9	76.4
07	54.0	78.4	60.3	85.4	85.8
08	46.8	65.4	33.6	79.9	76.4
09	50.0	71.6	47.4	79.9	82.4
10	51.6	69.1	42.2	78.5	81.1
Mean	**49.6**	**69.3**	**45.3**	**78.9**	**78.9**
SE	± 0.9	± 1.3	± 2.3	± 1.0	± 1.3
Shannon's information index (SI)					
01	0.36	0.45	0.23	0.54	0.54
02	0.40	0.45	0.22	0.55	0.55
03	0.36	0.42	0.21	0.52	0.50
04	0.35	0.43	0.22	0.51	0.52
05	0.34	0.49	0.26	0.57	0.58
06	0.31	0.47	0.25	0.57	0.55
07	0.37	0.52	0.30	0.61	0.61
08	0.34	0.45	0.17	0.56	0.56
09	0.35	0.48	0.25	0.57	0.59
10	0.35	0.47	0.22	0.56	0.57
Mean	**0.35**	**0.46**	**0.23**	**0.55**	**0.56**
SE	± 0.01	± 0.01	± 0.01	± 0.01	± 0.01

Both SI_{gen} and SI_{epigen} did not differ significantly between calcareous grassland and oat-grass meadow populations ($p = 0.245$ for SI_{gen}; $p = 0.115$ for SI_{epigen}; Table 4.5). Nevertheless, SI_{gen} was generally higher in calcareous grassland populations, while SI_{epigen} revealed higher values in oat-grass meadow populations (Table 4.5). Moreover, oat-grass meadow populations showed significantly higher m-subepiloci diversity ($p = 0.035$; Table 4.5). Additionally, environmental conditions concerning light, soil moisture, soil pH, and soil nitrogen differed significantly between calcareous grassland and oat-grass meadow populations ($p < 0.05$; Table 4.6).

Table 4.5: Differences of genetic and epigenetic diversity (Shannon information index) between calcareous grassland (CG) and oat-grass meadow (OM) populations (two-sided T-tests).

	Subpopulation			
	CG	**OM**	**p-value**	
AFLP	0.36	0.34	0.245	n.s.
MSAP				
all subepiloci	0.45	0.48	0.115	n.s.
h-subepiloci	0.23	0.24	0.685	n.s.
m-subepiloci	0.54	0.57	0.035	*
u-subepiloci	0.54	0.58	0.067	n.s.

Signif. codes: $0.01 < p \leq 0.05$ *; $p > 0.05$ n.s.

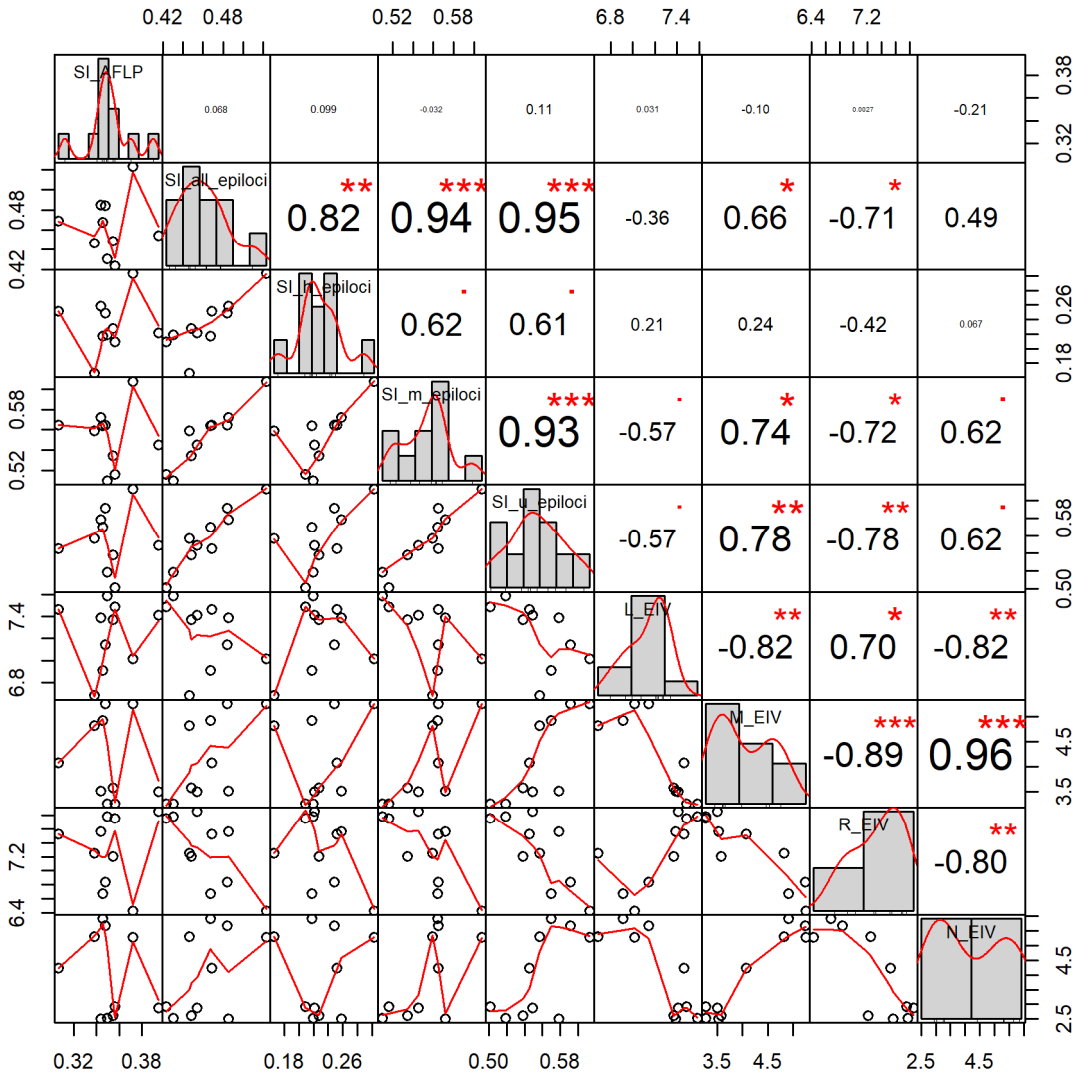
Table 4.6: Comparison of mean weighed Ellenberg indicator values (EIV) of light (L), soil moisture (M), soil reaction/pH (R), and soil nitrogen (N) between calcareous grassland (CG) and oat-grass meadow (OM) populations (two-sided T- and Wilcoxon-Mann-Whitney tests).

	Subpopulation			
	CG	**OM**	**p-value**	
L_EIV	7.45	7.04	0.032	*
M_EIV	3.43	4.86	0.012	*
R_EIV	7.63	6.94	0.022	*
N_EIV	2.69	5.29	0.008	*

Signif. code: $0.01 < p \leq 0.05$ *

SI_{gen} showed no correlation with environmental variables (light, soil moisture, soil pH, or soil nitrogen) (Figure 4.3). However, SI_{epigen} (all subepiloci, m-, and u-subepiloci) was significantly associated with soil moisture and soil pH (Figure 4.3). Thus, SI_{epigen} decreased with increasing drought and soil pH.

SI_{gen} revealed significantly lower values than SI_{epigen} ($p < 0.001$). Moreover, SI_{gen} and SI_{epigen} were not significantly correlated across populations ($\rho = -0.13$; $p = 0.733$).



Signif. codes: $p \leq 0.001$ ***; $0.001 < p \leq 0.01$ **; $0.01 < p \leq 0.05$ *; $p > 0.05$.

Figure 4.3: Correlation coefficients (Pearson correlation analyses) of Shannon information indices (SI) and environmental parameters represented by Ellenberg indicator values (EIV) for light (L), soil moisture (M), soil reaction/pH (R), and soil nitrogen (N).

Discussion

Genetic and epigenetic differentiation

Genetic differentiation levels were higher than epigenetic ones indicating that genetic variation may be more strongly structured than epigenetic variation (Lele et al., 2018). Some previous studies revealed the same results (Foust et al., 2016; Schulz et al., 2014), while other studies observed higher epigenetic than genetic differentiation levels (Herrera et al., 2017; Lira-Medeiros et al., 2010; Richards, Schrey, & Pigliucci, 2012).

Neither epigenetic nor genetic differentiation correlated with geographic distance among populations (IBD). Kloss et al. (2011) showed that an outcrossing breeding system as well as efficient dispersal of pollen and seeds may result in similar levels of genetic diversity over large spatial scales in common grassland species. Thus, spatial isolation did not play a major role for population differentiation in *T. pratense*.

However, even common and outbreeding species may reveal increased differentiation among populations through reduced abundance, spatial isolation, different land use regimes, and thus, lowered gene flow (Kloss et al., 2011). *T. pratense* populations showed higher genetic than epigenetic differentiation among habitat types. This result complies with the findings of Lele et al. (2018), who observed that genetic variation may play a more important role in habitat differentiation than epigenetic variation. Furthermore, genetic differentiation significantly correlated with habitat dissimilarity (IBH). Reisch and Poschlod (2009) observed that populations from mown and grazed habitats revealed higher genetic differentiation levels within the same geographic region, than similarly managed populations among different regions. Management practices like mowing and grazing differ strongly in intensity and time of application (Kloss et al., 2011; Reisch & Poschlod, 2009). Early mowing inhibits fruiting and seed production (Kloss et al., 2011) and thus, mown populations flower earlier than grazed ones (Reisch & Poschlod, 2009). These asynchronous flowering times inhibit gene flow, support genetic drift, and increase, therefore, genetic differentiation levels among contrasting habitats (Reisch & Poschlod, 2009). Thus, rather land use and related gene flow patterns than habitat type per se seem to shape genetic differentiation.

Genetic and epigenetic diversity

The comparison of genetic and epigenetic diversity among contrasting habitats revealed higher genetic diversity levels in calcareous grassland populations and higher epigenetic diversity levels in oat-grass meadow populations. These results comply with several studies, which surveyed different genetic and epigenetic diversity levels due to habitat type (Abratowska et al., 2012; Lira-Medeiros et al., 2010; Reisch & Poschlod, 2009; Wu et al., 2013).

Previous studies about genetic diversity patterns in common calcareous grassland (Lehmair, Pagel, Poschlod, & Reisch, *submitted*) and oat-grass meadow plant species (Pagel, Lehmair, Poschlod, & Reisch, *submitted*) observed a trend to higher genetic diversity levels in calcareous grassland populations. Within the study region, calcareous grasslands are still managed by migratory sheep herding and are, thus, exposed to elevated levels of disturbance by grazing and trampling. On the one hand, management induced disturbance may create suitable niches for seeds to germinate and seedlings to establish (Olff & Ritchie, 1998). On the other hand, grazing by sheep is an important vector for seed dispersal and enhances gene flow (Fischer et al., 1996; Rico et al., 2014a; Willerding & Poschlod, 2002). Therefore, management related disturbance and gene flow patterns seem to increase genetic diversity levels in calcareous grassland populations.

However, oat-grass meadow populations showed higher epigenetic diversity levels than calcareous grassland populations. The difference of epigenetic diversity between calcareous grassland and oat-grass meadow populations was significant only for m-subepiloci. Therefore, changes of hemimethylation in the CHG-context (m-subepiloci) may play a more important role for habitat adjustment than regulation of gene function in the CG-context (h-subepiloci). As mentioned above, the pattern and amount of DNA methylation in plants is sensitive to biotic and abiotic stressors (Herrera & Bazaga, 2013; Labra et al., 2002; Verhoeven et al., 2016). On the one hand, oat-grass meadows represent a comparatively homogenous habitat type with narrow ecological niches, since all species are simultaneously disturbed by mowing. Previous studies showed that an increase in epigenetic diversity may broaden ecological niches by expanding the species' potential to persist disturbance events (Medrano, Herrera, & Bazaga, 2014; Richards et al., 2012). On

the other hand, Pearson correlation analyses indicated that epigenetic diversity of *T. pratense* populations significantly decreased with increasing drought and soil pH. Therefore, challenging environmental conditions may affect epigenetic diversity in different ways.

Pearson correlation revealed no significant association between genetic diversity and environment. Pagel et al. (*submitted*) postulated landscape structure as key variable for genetic diversity of *T. pratense* populations in oat-grass meadows, while they could not observe any impact of local habitat quality. Therefore, genetic diversity of *T. pratense* may be affected more by landscape structure, related management, and/or gene flow patterns than by local environmental conditions.

However, several studies reported correlations between environmental factors and epigenetic characteristics of plant populations (Foust et al., 2016; Lira-Medeiros et al., 2010; Schulz et al., 2014). In this study, epigenetic diversity correlated significantly with soil moisture and soil pH. Thus, the epigenetic diversity of *T. pratense* populations seemed to be associated with environment, while genetic diversity was not. These results accompany with the assumption that DNA methylation and demethylation at a genome-wide scale are induced by environmental changes (Lira-Medeiros et al., 2010) and constitute an essential tool for plant species to react on biotic and abiotic environmental pressures (Labra et al., 2002; Verhoeven et al., 2016). Moreover, epigenetic variation is supposed to increase under challenging environmental conditions (Dowen et al., 2012; Herrera et al., 2012; Verhoeven et al., 2010). Labra et al. (2002) emphasized that different plant species may show varying DNA methylation patterns depending on the kind of challenging environmental conditions. Thus, the assumption that epigenetic diversity grows under challenging environmental conditions should not be generalised across all species. In this study, epigenetic diversity decreased under drought. This result was in line with the study of Davis (1991), who observed that *T. pratense* did not perform well under drought stress. Furthermore, Labra et al. (2002) postulated that active methylation or demethylation of cytosine could occur dynamically in response to water stress (Dowen et al., 2012). Thus, epigenetic diversity of *T. pratense* populations may decrease with increasing drought. Additionally, epigenetic diversity decreased with increasing soil pH. Soil pH influences the

amount of plant available nutrients. Since *T. pratense* is a nitrogen-fixing legume (Carlsson & Huss-Danell, 2003), its performance is sometimes limited by plant accessible phosphorus (Davis, 1991). In calcareous soils, phosphorus is bound to calcium phosphate (Frossard et al., 1995) and thus, not plant available. The calcareous grasslands in our study revealed the highest soil pH. Therefore, *T. pratense* populations may show limited productivity and decreased epigenetic diversity as reaction to phosphorus limitation. However, the correlation with soil moisture and soil pH was not significant for h-epiloci indicating that the regulation of gene function by (de-)methylation in the CG-context may not be an issue for adaptation to different environmental conditions.

Previous studies observed higher levels of epigenetic than genetic diversity especially in natural plant populations (Foust et al., 2016; Herrera & Bazaga, 2010; Lele et al., 2018; Lira-Medeiros et al., 2010). In *T. pratense*, epigenetic diversity was even significantly higher than genetic diversity indicating that these natural populations seem to vary more in DNA methylation than in DNA sequence (Hirsch et al., 2012).

Furthermore, neither correlation nor simple Mantel tests revealed a significant association of epigenetic with genetic diversity or distance. In this context Richards (2006) defined three classes of epigenetic variation at a given locus: (i) obligatory: epigenotype is strictly determined by genotype, (ii) facilitated: epigenotype depends on both genotype and environmental context, or (iii) pure: epigenotype is created by environmental context. On the one hand, Foust et al. (2016) stated that studies which cannot sample the entire genome may miss genomic elements or genes that are involved in or affected by DNA methylation. On the other hand, they considered the application of molecular markers in natural populations as a useful tool to identify epigenetic structures, which are not explained by DNA sequence. Thus, we assume that epigenetic and genetic diversity may differ in their ecological and evolutionary implications (Herrera & Bazaga, 2010; Jablonka, 2013) and classify the epigenetic variation of *T. pratense* populations as rather facilitated or pure than obligatory. This finding is in accordance with the results of previous studies on wild plants, which also observed epigenetic variation to be largely autonomous from genetic variation (Herrera & Bazaga, 2016; Paun et al., 2010).

Conclusions

Our results revealed an impact of different environmental conditions on genetic and epigenetic variation. Genetic variation was affected by habitat specific environmental conditions induced by management related disturbance as well as gene flow patterns. Epigenetic variation was driven by challenging environmental conditions in two ways. It increased with rising necessity for niche establishment, but decreased under drought and high pH, the latter potentially resulting in phosphorus limitation.

Nevertheless, MSAP marker reveal only a limited number of anonymous loci, which are difficult to link to functional genomic elements. Therefore, future studies should apply next-generation based bisulphite sequencing approaches to evaluate the effects of challenging environmental conditions on methylation patterns more precisely (Lele et al., 2018).

CHAPTER 5

GENERAL DISCUSSION AND CONCLUSIONS

Pulsatilla vulgaris Mill. s. str.

Semi-natural grasslands represent biodiversity hotspots within the agricultural landscape of Europe (Duelli & Obrist, 2003; Eriksson et al., 1995; Habel et al., 2013). Extensive management with grazing or mowing shaped the man-made habitats revealing important ecosystem services besides outstanding species-richness (Hopkins, 2009; Pärtel et al., 2005; WallisDeVries et al., 2002). Intensification of agricultural practices with increased fertilization, drainage, ploughing, and cutting numbers or abandonment with subsequent succession (Muller et al., 1998) changed and still change local environmental conditions (van der Meer et al., 2014). The abandonment of traditional land use practices resulted, moreover, in a vast area decline (Bakker, 1989; Poschlod et al., 2005; Poschlod & Bonn, 1998) and thus, in fragmentation (Fischer & Stöcklin, 1997; Helm et al., 2006; Picó & Van Groenendael, 2007). Populations of many plant species consequently suffer from reduced probabilities of gene flow, increased genetic drift, lowered genetic variation, and increased extinction risk (Aguilar et al., 2008; Ouborg et al., 2006; Spielman et al., 2004). Hence, the number of plant and animal species drastically declined during the last decades (Hallmann et al., 2017; Pimm et al., 2014; Seibold et al., 2019).

Numerous conservation strategies were developed and initiated especially since the Convention on Biological Diversity (CBD, 1992) to counteract this biodiversity decline. Nevertheless, both *ex situ* and *in situ* conservation strategies attach too little or no importance to the conservation of plant genetic resources (Laikre et al., 2010; Ramanatha Rao & Hodgkin, 2002).

The present study identified, therefore, potential drivers of genetic diversity in populations of six common plant species in two semi-natural grassland habitats. Moreover, the relevance of and impact factors on genetic and epigenetic variation were compared between two contrasting habitats.

Plant genetic diversity and differentiation

Genetic diversity and differentiation patterns depend on species' pollination, mating, and dispersal systems (Oostermeijer et al., 1996; Schoen & Brown, 1991). All study species were mainly pollinated by insects, except for *L. catharticum* (Kühn et al., 2004). *A. cynanchica* and *C. rotundifolia* populations on calcareous grasslands revealed, therefore, only slightly

higher levels of genetic diversity than *A. sylvestris*, *F. ulmaria*, and *S. pratensis* populations on litter meadows. *L. catharticum*, which is mostly considered as self-pollinated annual, exhibited the lowest levels of genetic diversity. The mean genetic diversity of our study species complied, moreover, with genetic diversity levels previously observed for insect and self-pollinated species, respectively (Reisch & Bernhardt-Römermann, 2014).

Sufficient gene flow at the time of founding and afterwards might reduce the effects of habitat age (Vandepitte et al., 2010). Dispersal by hay, sowing, and seedling transfer from ancient sites as well as permanent pollen and seed exchange through pollinators, agricultural machines, and sheep (Poschlod & WallisDeVries, 2002; Stebler, 1898) led to substantial levels of gene flow over long time periods and large distances (Fischer et al., 1996; Poschlod, 2017; Poschlod et al., 1998). Hence, both calcareous grassland and litter meadow populations revealed similar levels of genetic diversity and no differentiation among ancient and recent sites.

Moreover, weak levels of differentiation and comparatively low Φ_{PT} values among populations led to the assumption that spatial distances among populations still seem to allow gene flow by pollen and seed dispersal (Kloss et al., 2011; Neel, 2008). Nowadays, litter meadows generally apply as highly fragmented habitats. Nevertheless, the here investigated *A. sylvestris*, *F. ulmaria*, and *S. pratensis* populations were not isolated by distance. Gene flow is high, since all three species are pollinated by many different insect species (Kühn et al., 2004) and seeds are sufficiently dispersed and exchanged by mowing machines (Strykstra et al., 1997). Moreover, remnant litter meadows cover a significantly smaller region (< 35 km) than remaining calcareous grasslands (< 100 km). Thus, calcareous grassland populations examined here revealed lower levels of gene flow. On the one hand, pollinating insects may rarely travel distances larger than 1 km (Kwak et al., 1998; Steffan-Dewenter & Tscharntke, 2002). On the other hand, increasing abandonment of migratory sheep farming may limit the probability for seed dispersal among remote sites. Therefore, the insect pollinated as well as ecto- and/or endozoochorously dispersed *A. cynanchica* and *C. rotundifolia* populations (Kühn et al., 2004; Poschlod et al., 2003) were isolated by distance. The mainly self-pollinated *L. catharticum* (Kühn et al., 2004) did not reveal any isolation by distance.

Drivers of genetic diversity

Variables driving genetic diversity patterns of calcareous grassland and litter meadow species mainly differed due to habitat affiliation – with two exceptions (Figure 5.1). First, genetic diversity of *A. cynanchica* and *S. pratensis* populations decreased with increasing population size. According to the results of previous studies (Giles & Goudet 1997; Jacquemyn *et al.* 2004; Münzbergová *et al.* 2013) historical changes in land use and landscape structure may reveal a major impact on present genetic diversity patterns. Hence, this result indicates a time lag between habitat loss, fragmentation, and their consequences on genetic diversity (extinction dept) (Helm et al., 2006). Second, the present total area of surrounding grasslands revealed a positive impact on both *L. catharticum* and *F. ulmaria* populations by increasing their probability for gene flow (Lonn & Prentice, 2002; Slatkin & Voelm, 1991).

Comparatively moderate levels of gene flow may reveal a positive impact on genetic diversity levels, while 'too low' and even 'too high' levels of gene flow may promote outbreeding depression and/or genetic 'swamping' (Bradshaw, 1984). Thus, present connectivity either increased genetic diversity levels of *A. cynanchica* populations on highly fragmented calcareous grasslands or decreased genetic diversity levels of *F. ulmaria* populations on 'over-connected' litter meadows. Nowadays, remnant litter meadows occur over a comparatively small spatial scale and are managed by a few conservation managers (personal communication). Hence, seeds are transported well among sites by mowing machines (Strykstra et al., 1997) leading to an impoverished gene pool by genetic 'swamping'. In former times, litter meadows were cultivated by many different farmers (personal communication) and thus, past connectivity revealed a positive impact on genetic diversity levels of *F. ulmaria* populations.

A similar pattern could be observed in terms of the intermediate disturbance hypothesis (Connell, 1978). Anthropogenic management led to both an increase of genetic diversity in *A. cynanchica* populations around present settlements and a decrease of genetic diversity in *C. rotundifolia* populations close to historic settlements. Moreover, the past total area of surrounding calcareous grasslands revealed also a negative impact on the genetic diversity in *A. cynanchica*. The populations of both species may still reflect the

impact of periodic overgrazing and exceeding levels of disturbance and gene flow in the past. Nevertheless, calcareous grassland species depend on regular, moderate disturbance by selective grazing and trampling by cattle, sheep, and goats (Dierschke & Briemle, 2002; Olff & Ritchie, 1998). Thus today, *A. cynanchica* populations seem to be disturbed and connected at an intermediate level around present settlements.

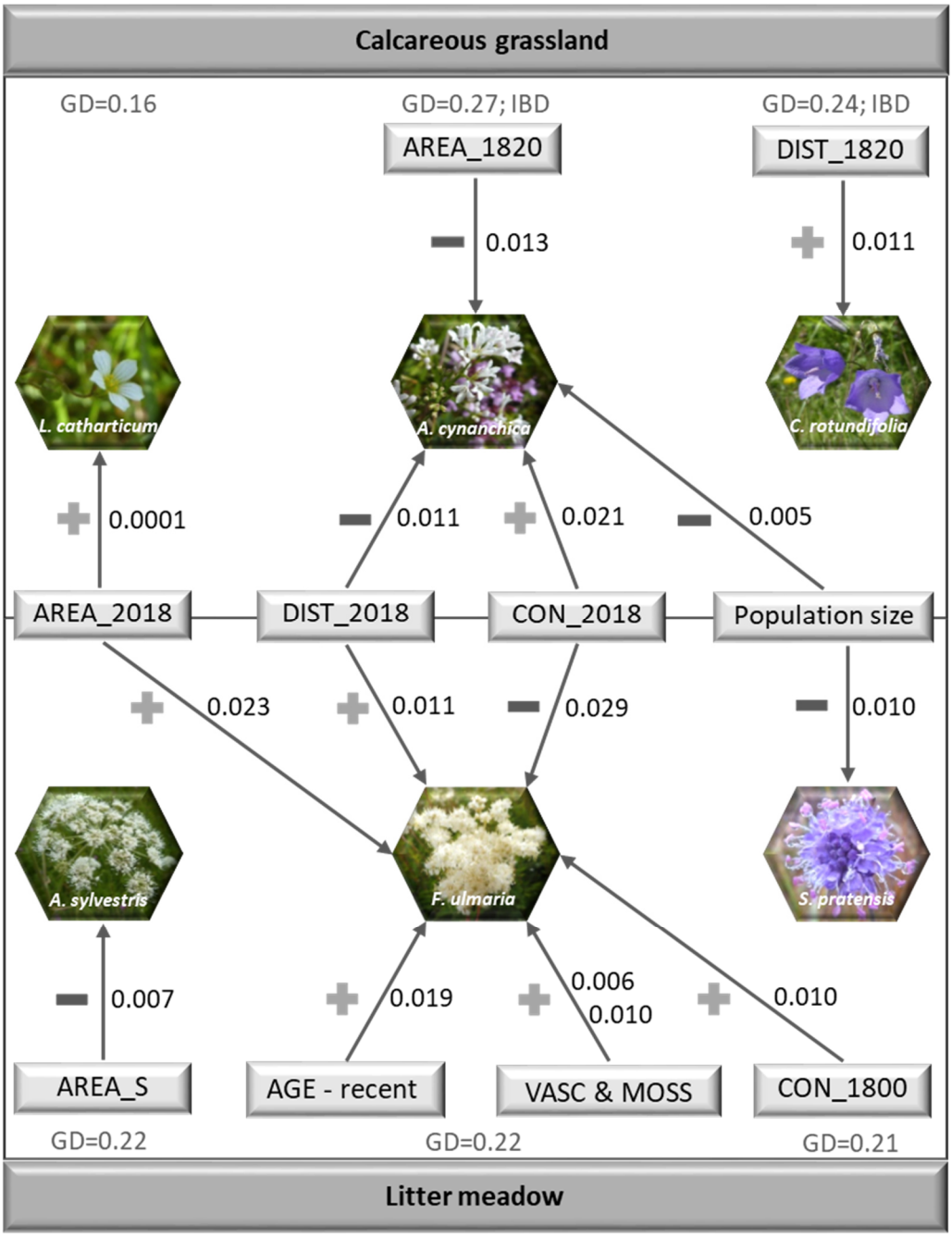
However, less disturbance by anthropogenic land use led to an increase of genetic diversity in *F. ulmaria* on litter meadows far off present settlements. Litter meadows are traditionally mown once a year in late autumn with waiver of additional fertilizer application (Poschlod, 2017). Hence, characteristic litter meadow species may suffer from enhanced levels of disturbance by intensified agricultural land use with fertilizer application and increased cutting numbers today (Dierschke & Briemle, 2002).

Measures of habitat quality, such as cover of vascular plants and mosses affected genetic diversity levels of *F. ulmaria* and *S. pratensis*. Therefore, we assume that species' efforts for successful germination and subsequent establishment may play a more important role in well-connected litter meadows than in widely distributed and highly fragmented calcareous grasslands.

In general, genetic diversity of all investigated species was driven by different impact factors in various ways (Figure 5.1). Species, such as *C. rotundifolia*, *L. catharticum*, *A. sylvestris*, and *S. pratensis* were affected by only one key variable, while genetic diversity of *A. cynanchica* and *F. ulmaria* was driven by an interaction from four to six different parameters.

Nevertheless, gene flow and/or disturbance mechanisms steadily underpinned the drivers of genetic diversity. Nowadays, abandonment of migratory herding limits seed dispersal and disturbance by grazing animals (Fischer et al., 1996; Olff & Ritchie, 1998; Willerding & Poschlod, 2002). Comparatively large distances among fragmented calcareous grasslands reduce, moreover, gene flow by pollinating insects (Steffan-Dewenter & Tscharntke, 2002). Restricted levels of gene flow and/or increased genetic drift decrease genetic diversity. Therefore, high levels of gene flow by pollinators and traditional animal husbandry seem to be the key variables for genetic diversity conservation in highly fragmented calcareous grasslands these days. However, the distribution of remnant litter

meadows is spatially limited today. Although litter meadows are also considered as highly fragmented habitats, sites investigated here were sufficiently connected through high levels of gene flow by pollinators and seed dispersal by mowing machines. Therefore, litter meadow species seem to suffer more from disturbance by land use intensification and thus, missing germination niches, than from limited gene flow nowadays.



GD, mean Nei's gene diversity per species; IBD, isolation by distance; AGE, habitat age; AREA_S, area size [ha]; AREA_2018, present total area of calcareous grasslands/wet meadows [ha]; DIST_1820/DIST_2018, past and present distance to the nearest settlement [km]; CON_1800/CON_2018, past and present connectivity; VASC, cover of vascular plants [%]; MOSS, cover of mosses [%]

Figure 5.1: Impact factors on genetic diversity in calcareous grassland (upper square) and litter meadow species (lower square). The estimates are given in black. Mean genetic diversity levels per species and isolation by distance are displayed in grey. '+' symbolizes a positive association, '-' a negative one.

Genetic reserves

The findings obtained could be applied to protect plant genetic resources of semi-natural grasslands with high conservation value. The conservation of 'crop wild relatives' (CWR) by the 'European Cooperative Programme for Plant Genetic Resources' (ECPGR) in genetic reserves (Maxted et al., 2015) was already conducted on an individual, national, and global scale (Maxted & Kell, 2009). Nevertheless, a conservation approach for genetic resources of non-CWR species, such as grassland species, is still missing. Therefore, the concept of genetic reserves should also be used to monitor and protect plant genetic resources of non-CWR species within defined areas (*in situ*). These areas may act as donor sites for habitat creation, restoration, or diversity enhancement using natural populations and native seeds (Hopkins, 2009). Moreover, the conservation of sites with low value of agricultural production, as *in situ* resources for genetic variation, may increase both the conservation status of these sites and the economic benefits for their owners (Hopkins, 2009).

Countless guidelines were already formulated for efficient genetic reserve identification, establishment, and maintenance (e.g. Maxted & Kell 2009; Iriondo *et al.* 2012; Frese, Anna & Kik 2014; Maxted *et al.* 2015). In general, the development of a clear strategic plan is obligatory before initiating *in situ* reserves. Hence, target taxa should be selected and objectives for conservation activities should be determined. The core objective for genetic resource conservation is to protect the maximum range of genetic diversity within a minimal set of sites (Maxted et al., 2000). Molecular markers should be used to determine the dimension of the target gene pool and to evaluate genetic variation patterns of eligible sites. Conservation goals should, moreover, include the selection and establishment of mutual complementary reserves per target species (Maxted et al., 2000; Rubio Teso & Iriondo, 2019). Sites should be selected above the widest possible range of ecogeographic conditions colonized by the target species (Maxted et al., 2000) containing genetically differentiated units with locally adapted genotypes (Picó & Van Groenendael, 2007). These sites represent important targets for conservation providing a measure of buffering against threats from environmental and anthropogenic stochasticity (Neel & Cummings, 2003). This approach may, therefore, enhance the conservation of a

representative part of the entire gene pool and permit the conservation of as many ecotypes as present in the target species (Maxted et al., 2000).

In practice, the questions of 'how many' and 'which populations' should be answered to maintain the species' future evolutionary potential and probability of persistence at the best (Barrett & Kohn, 1991; Ellstrand & Elam, 1993; Newman & Pilson, 1997). It is generally accepted that a single population could not represent the extent, distribution, and structure of a species' genetic variation. Thus, the best way to cover the maximal possible genetic variation is by a subset of populations (Neel & Cummings, 2003). Moreover, conservation decisions should include both genetic diversity and differentiation (Neel & Cummings, 2003) as well as potential impact factors (Ramanatha Rao & Hodgkin, 2002). In the absence of genetic variation, species and populations lack the ability to evolve against the background of challenging environmental conditions (McKay et al., 2005). Hence, genetic variation, representing both genetic diversity and genetic differentiation, constitutes the basis of evolutionary change, fitness, and survival (Ramanatha Rao & Hodgkin, 2002). Plant species' reactions to biotic and abiotic environmental conditions differ and thus, key variables affecting genetic structure, variation patterns, and distribution of alleles should be clearly understood (Ramanatha Rao & Hodgkin, 2002; Rubio Teso & Iriondo, 2019). Due to limited time and/or funding, this kind of knowledge is scarce and usually not considered in genetic reserve establishment (Keller et al., 2015; Neel & Cummings, 2003).

Nevertheless, the results obtained in this thesis could be applied exemplarily to conceive a promising approach for genetic reserve identification. As first step, a modified approach of Neel and Cummings (2003) and Whitlock *et al.* (2016) could be implemented to answer the question of 'how many populations' (Figure 5.2). Thus, the highest value measured for genetic diversity is set as 100 % and populations are drawn randomly from the total set of populations investigated per species. The resulting saturation curve indicates the modal diversity for each number of sampled populations. According to the tenth conference of the parties to the Convention on Biological diversity (CBD, 2010) the minimal set of conservation sites should represent 70 % of the highest measured genetic diversity level (Figure 5.2). However, we would recommend to aim for 90 – 99 % where

possible. A second step should determine 'which populations' by selecting the populations with the highest Φ_{PT} value (∑ 'genetic distance to all other investigated populations') to represent the most differentiated populations. In a third step previous findings about drivers of genetic variation, such as landscape structure, habitat quality, or population size should be included to ensure efficient long-term conservation. These steps may be a first approach for sufficient genetic reserve identification and conservation, but efficient genetic reserve establishment and maintenance will need more actions.

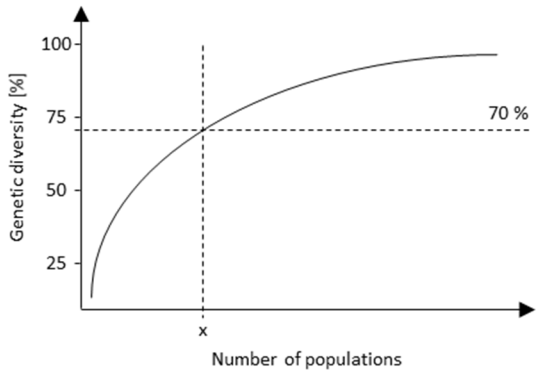


Figure 5.2: Effects of population number on genetic diversity [%], with 100 % corresponding to the highest value measured for genetic diversity. Populations are drawn randomly from the total set of each species. The saturation curve indicates, therefore, the modal diversity for each number of sampled populations. The dotted black lines show the interface at 70 % of the highest value measured for genetic diversity, which is covered by x populations.

Effective conservation strategies need to combine *in situ* and *ex situ* approaches (Maxted et al., 2000). *In situ* conservation ensures the preservation of genetically adapted populations continually allowing natural evolution to shape genetic variation (Greene et al., 2014). Therefore, the large quantity of resources expended on reserve establishment will be wasted and, what is more important, the genetic resource as a whole will be irretrievably lost as soon as *in situ* protected material disappears (Maxted et al., 2000). Populations could be restored from previously collected *ex situ* material, but costs are particularly high (Maxted et al., 2000). *Ex situ* accessions represent the inherent diversity of *in situ* populations at the time of sampling and thus, a static genetic snapshot with potentially already lost alleles from *in situ* populations (Greene et al., 2014). Nevertheless,

ex situ accessions could become inbred, loose adaptation to their source environment (Schoen & Brown, 2001), or diverge genetically from their source populations (Lauterbach et al., 2012). Thus, extensive research is required to ensure the successful establishment, survival, and reproduction of reintroduced material (Maxted et al., 2000).

Moreover, genetic reserves should comply a minimum set of quality standards as proposed by Iriondo *et al.* (2012) to ensure effective long-term conservation after establishment. Additionally, threatening processes, such as climate change or devastating changes by human or natural interventions leading to an interruption or even termination of natural processes and/or traditional management, should be mitigated as far as possible (Maxted et al., 2000; Rubio Teso & Iriondo, 2019). The most promising way to protect plant genetic resources sustainably and efficiently is to establish genetic reserves within or linked to already protected areas (Iriondo et al., 2012; Maxted et al., 2000). Costs of genetic reserve establishment and maintenance could be reduced applying already existing conservation laws and management plans (Maxted et al., 2000; Rubio Teso & Iriondo, 2019). Nevertheless, sites with high conservation priority, which are not embedded in protected areas so far, should also be considered establishing future networks of genetic reserves.

In general, populations within protected areas are not actively monitored and thus, deleterious environmental changes are often overseen and not counteracted. Therefore, genetic reserves should be actively monitored, managed, and protected to support sustainable populations of the target species as well as maintenance of the ecosystems (Maxted et al., 2000). Farmers, who implement historical knowledge about traditional management in conservation strategies (Maxted et al., 2000), represent the most important component of plant genetic resource conservation in grasslands. Adequate monitoring of genetic resources by scientists or NGOs constitutes another key component. Therefore, the transfer of knowledge between farmers, NGOs, and scientists appears crucial for conservation measures (Maxted et al., 2000).

Strengths, limitations, and perspectives

The selection of target species constitutes a highly controversial issue in genetic resource conservation. Rare plant species generally show high conservation priority, although widespread species are rapidly declining through connectivity loss and potential inbreeding depression today (Whitlock et al., 2016). Hence, species, which are still classified as common, may suffer rather more from population loss than rare species with current stable distribution patterns (Whitlock et al., 2016). Common, but habitat specific plant species could, moreover, function as umbrella species for genetic resource conservation of entire ecosystems (Roberge & Angelstam, 2004). Therefore, we investigated habitat specific and comparatively common plant species to get an overview over potential drivers of genetic diversity in calcareous grasslands and litter meadows. This thesis showed that various drivers differently shape plant genetic variation depending on species affiliation. Based on these results plant genetic resources could be protected on species level, but a more comprehensive scientific approach will be necessary to protect plant genetic resources above species level. Hence, a multi-species approach on a greater extent may provide information about potential least common denominators driving genetic variation, e.g. for species groups with similar live history traits or even entire ecosystems.

Study regions investigated here were spatially limited. Geographic differences in the distribution of genetic variation are very common and could not be separated from ecologically determined variation (Ramanatha Rao & Hodgkin, 2002). Populations located in different geographic regions may vary in the number of alleles, the identity of those alleles, and their effect on population characteristics (Ramanatha Rao & Hodgkin, 2002). Moreover, distribution patterns of several species changed significantly during the past century (Picó & Van Groenendael, 2007). Therefore, the geographic structure of genetic variation should be tested on a larger spatial scale to develop a comprehensive conservation plan, to protect ecological and evolutionary processes generating and maintaining biodiversity, and thus, to improve genetic resource conservation (Whitlock et al., 2016).

Neutral markers (e.g. AFLP) are often not regarded as reliable indicators for populations' adaptive potential to ecological traits (McKay et al., 2005). Nevertheless, they

predict the impact of different variables on genetic variation and display patterns of gene flow and genetic drift (McKay & Latta, 2002; Vellend, 2005). Thus, neutral markers enhance our understanding of genetic variation patterns and offer a valuable method to develop and evaluate conservation guidelines and strategies (Whitlock et al., 2016).

The results of Whitlock *et al.* (2016) stressed, moreover, that conservation targets for genetic resource conservation should also include rare allelic variation patterns. The focus of this thesis was set on genetic variation patterns to avoid extreme complex linear regression models. Nevertheless, rare allele frequencies as well as their potential drivers should be considered for genetic resource conservation to facilitate species' reaction to changing environmental conditions (Loewe & Hill, 2010).

Traditionally, the reaction of plant species to changing environmental conditions was exclusively based on genetic variation patterns (Wu et al., 2013). However, numerous studies linked the adaptive potential of populations to epigenetic variation during the last decades (e.g. Bossdorf et al., 2008; Herrera & Bazaga, 2011; Jablonka & Raz, 2009; Lira-Medeiros et al., 2010; Paun et al., 2010; E. J. Richards, 2006; Schulz et al., 2013, 2014; Wendel & Rapp, 2005; Wu et al., 2013), since natural variation occurs not only in the DNA sequence, but also at the epigenetic level (Richards et al., 2010). Thus, genetic (AFLP) and epigenetic (MSAP) variation patterns of *T. pratense* were compared between two contrasting habitats. On the one hand, genetic variation was affected by habitat specific environmental conditions induced by land use related disturbance and gene flow patterns. On the other hand, epigenetic variation was directly driven by challenging local environmental conditions. Additionally, genetic and epigenetic variation were not interdependent suggesting that epigenetic variation (e.g. by DNA methylation) may be the key component for plant species' response to challenging environmental conditions (Bossdorf et al., 2008). Knowledge about the impact of epigenetic variation on non-model plant species is still scarce (Abratowska, Wasowicz, Bednarek, Telka, & Wierzbicka, 2012; Herrera & Bazaga, 2010; Lira-Medeiros et al., 2010; Wu et al., 2013). However, epigenetic markers are potentially heritable and can, therefore, be under selection or even might impact evolution (Jablonka & Raz, 2009; Richards, 2006). Before the potential role of epigenetic variation on plant adaptation can be assessed, questions about the magnitude,

structuring within and among natural populations, and potential autonomy in relation to the underlying genetic code, should be addressed (Kalisz & Purugganan, 2004; Richards, 2006; Wendel & Rapp, 2005). However, accepting the hypothesized role of epigenetic variation, directly or indirectly affecting the course of evolution in plants (Herrera & Bazaga, 2010), epigenetic mechanisms represent the basis of all three levels of biodiversity defined by the CBD (1992) (Figure 1.1). Profound knowledge of epigenetic mechanisms may, therefore, provide a nuanced understanding of mechanisms underlying plant reactions to changing environmental conditions (Foust et al., 2016). Hence, the consideration of epigenetic variation patterns becomes absolutely necessary for effective genetic resource conservation.

Conclusions

Genetic variation represents the most fundamental level of biodiversity (May, 1994) and thus, there is a pressing need to understand, enhance, protect, and use genetic resources sensibly. This thesis provided important insights in key interactions affecting genetic variation patterns. The investigation of three species per habitat type revealed already rough trends on how genetic variation is driven on ecosystem level, although all impact factors performed species dependent. Among fragmented populations, e.g. on calcareous grasslands, the establishment of sub- or stepping stone populations may allow moderate levels of gene flow by pollinators (Kimura & Weiss, 1964; Levins, 1969; Wright, 1969). Moreover, moderate levels of migratory herding should be supported to ensure seed dispersal, periodic disturbance, and nutrient removal by grazing animals (Ellenberg, 1996; Fischer et al., 1996; Olff & Ritchie, 1998; Willerding & Poschlod, 2002). In well connected sites, e.g. on litter meadows, germination niches should be promoted by keeping levels of agricultural management and disturbance low. To sum up, genetic variation in populations of both habitat types depended on human interactions in accordance to past and present landscape structures. Nevertheless, more research is needed to understand genetic variation patterns at levels of species groups and ecosystems more accurately.

Besides genetic variation, epigenetic variation should be taken into account studying plant genetic resources. This thesis emphasised epigenetic variation as potential

key component for rapid response to and survival of challenging environmental conditions. Exposure to different environmental conditions may impact epigenetic variation patterns, which seem to be under selection and even might impact evolution (Jablonka & Raz, 2009; Richards, 2006). These findings clearly suggest that epigenetic mechanisms could add a new dimension of complexity to the diversity and evolutionary potential of natural populations (Richards et al., 2010). Hence, both genetic and epigenetic variation patterns should be investigated before establishing genetic *in situ* reserves.

A profound systematic scientific approach should, moreover, include information about systematics, *ex situ* and *in situ* conservation methods, past and present landscape ecology, and conservation biology (Ramanatha Rao & Hodgkin, 2002). In this context, international cooperation or joint ventures may improve the access to already existing knowledge, facilitate information exchange (especially of molecular data), and simplify genetic resource conservation.

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1 SUPPLEMENTARY MATERIAL - DNA EXTRACTION, AFLP AND MSAP ANALYSES

DNA extraction

DNA was extracted from sampled leaf material following the CTAB protocol from Rogers and Bendich (1994) as modified by Reisch (2007).

A ball mill (Retsch MM400) was used to grind 10 to 15 mg fresh or frozen leaf material with a steel ball in a 2.0 ml vessel. A preheated solution consisting of 1.0 ml CTAB buffer (100 mmol/L Tris, pH 9.5; 20 mmol/L EDTA, pH 8.0; 1.4 mol/L NaCl; CTAB 2.0 %; PEG-6000 1.0 %; SIGMA) and 2.5 µl β-mercaptoethanol (Merck KGaA) was added per sample. Samples were incubated for 30 min at 74 °C and turned over in 10 min intervals.

After a cooling phase of 10 min, 700 µl chloroform-isoamyl alcohol solution (24:1; 5 °C; Fisher Chemicals) was added for the first protein precipitation. Samples were mixed and centrifugalized at 4 °C for 10 min at 10,000 g. For the second protein precipitation, 500 µl supernatant was removed and mixed with 500 µl chloroform-isoamyl alcohol solution (24:1; 5 °C; Fisher Chemicals) in a 1.5 ml vessel. The samples were centrifugalized at 4 °C for 10 min at 14,000 g.

For the DNA precipitation, 300 µl supernatant was removed and gently mixed with 300 µl isopropyl alcohol (VWR Chemicals) in a 1.5 ml vessel. The samples were incubated for 30 min at room temperature and afterwards centrifugalized at 4 °C for 5 min at 10,000 g. After decanting the isopropyl alcohol, the pelleted DNA was washed once by adding 500 µl ethanol (70 %; 5 °C; Sigma ALTRICH) and centrifugalized at 4 °C for 15 min at 14,000 g. Afterwards the DNA pellet was dried in a vacuum concentrator (Eppendorf).

The pelleted DNA was redissolved in 100 µl TE-buffer (10 mmol/L Tris, pH 8.0; 0.1 mmol/L EDTA, pH 8.0) over night. All DNA samples were diluted to the same level of 7.8 ng DNA per µl H_2O after measuring the concentration of genomic DNA with a spectrophotometer.

AFLP analyses

The following AFLP analyses were conducted in accordance to the protocol of Beckmann Coulter (Bylebyl et al., 2008; Reisch, 2008).

Double strand DNA adapters were generated in a 0.2 ml reaction vessel. After adding equal volumes of both single strands of *EcoRI* and *MseI* adaptors (Biomers), 5 min heating at 95 °C was followed by a final 10 min step at 25 °C.

Restriction of 6.4 µl diluted genomic DNA (7.8 ng/µl) and ligation of DNA adaptors were performed in one step. We added 3.6 µl containing 2.5 U *Eco*RI (Thermo Scientific), 2.5 U *MseI* (Thermo Scientific), 0.1 µmol/L *EcoRI* as well as 1 µmol/L *MseI* adapter pair, 0.5 U T4 DNA ligase with its corresponding buffer (Thermo Scientific), 0.05 mol/L NaCl and 0.5 µg BSA (BioLabs/NBA). Following an incubation for 2 h at 37 °C and a subsequent enzyme denaturation step at 70 °C for 15 min, the products were diluted 10 fold with 1:10 TE buffer (20 mmol/L Tris-HCl, pH 8.0; 0.1 mmol/L EDTA, pH 8.0).

The preselective amplification was performed with 1 µl diluted DNA restriction-ligation product and 4 µl core mix, consisting of preselective *Eco*RI and *MseI* primers (Biomers) with a single selective nucleotide (*EcoRI*-A and *MseI*-C) and an AFLP core mix containing 1× Buffer S, 0.2 mmol/L dNTPs, and 1.25 U Taq-Polymerase (PeqLab). The PCR started at 94 °C for 2 min, followed by 30 cycles of 20 s denaturation at 94 °C, 30 s annealing at 56 °C and 2 min elongation at 72 °C. Then, 2 min at 72 °C finally ended the elongation period and 30 min at 60 °C with a cool down to 4 °C completed the PCR run. After this, the products were diluted 20 fold with 1:10 TE buffer.

After the screening of 36 to 42 primer combinations with eight randomly selected individuals, three primer combinations were chosen per species for further analysis (Table S2.5, Table S3.5 & Table S4.2). The selective amplification was performed in a total reaction volume of 5 µl, consisting of 0.75 µl diluted preselective amplification product and 4.25 µl core mix, containing 0.05 µmol/L selective *Eco*RI (Biomers) primers, 0.25 µmol/L *Mse*I (Biomers) primers, and AFLP core mix with 1× Buffer S, 0.2 mmol/L dNTPs, 1.25 U Taq-Polymerase (PeqLab). *EcoRI* primers were labelled with three different fluorescent dyes for fragment detection (Beckman dye D2, D3, and D4). Following PCR parameters were chosen: 2 min at 94 °C; then 10 cycles of 20 s denaturation at 94 °C, 30 s annealing at 66 °C

(temperature was reduced every subsequent step by 1 °C), and 2 min elongation at 72 °C; then additional 25 cycles of 20 s denaturation at 94 °C, 30 s annealing at 56 °C, and 2 min elongation at 72 °C, completed by a following 30 min step at 60 °C and a cool down to 4 °C. Selective PCR products were diluted as shown in Table S2.5, Table S3.5 and Table S4.2 with 1:10 TE buffer.

The amplified selective PCR products of each individual (5 µl) were added to a stop solution, consisting of 2 µl sodium acetate (3 mol/L, pH 5.2), 2 µl Na_2EDTA (100 mmol/L, pH 8.0), and 1 µl glycogen (Roche). Precipitation of DNA was performed by adding 60 µl of ice-cold ethanol (96 %; -20 °C), followed by an immediate shaking and subsequent centrifugation at 4 °C for 20 min at 14,000 g. The pelleted DNA was washed once by adding 200 µl ice-cold ethanol (70 %; -20 °C) and centrifugalized at the latter conditions. Afterwards, the DNA pellet was dried in a vacuum concentrator (Eppendorf). The pelleted DNA was redissolved in a mixture of 24.8 µl Sample Loading Solution (Beckman Coulter) and 0.2 µl CEQ Size Standard 400 (Beckman Coulter).

The fluorescence-labelled DNA fragments were separated by capillary gel electrophoresis according to their size using an automated capillary electrophoresis machine (GeXP, Beckmann Coulter). Results were examined with the GeXP software (Beckmann Coulter, USA). The received data were exported into three curve-files, each representing one primer pair. These virtual gels were analysed manually using the software Bionumerics 4.6 (Applied Maths, Kortrijk, Belgium). Only strong and clearly defined fragments were taken into account for further analyses, while samples without clear banding pattern were repeated or ultimately excluded.

MSAP analyses

The MSAP analyses were based on the standard AFLP protocol of Beckmann Coulter (Bylebyl et al., 2008; Reisch, 2008) modified by Schulz *et al.* (2013).

Double strand DNA adapters were generated in 0.2 ml reaction vessels. Equal volumes of both single strands of *EcoRI*, *HpaII*, and *MspI* adaptors (Biomers) were merged by a 5 min heating at 95 °C and a final 10 min step at 25 °C. All following steps were performed in two separate runs for each of the methylation-sensitive restriction enzymes *HpaII* and *MspI*. Restriction of 6.4 µl diluted genomic DNA (7.8 ng/µl) and ligation of DNA adaptors were performed in one step. We added 3.6 µl containing 2.5 U *EcoRI* (Thermo Scientific), 2.5 U *HpaII* or *MspI* (Thermo Scientific), 0.1 µmol/L *EcoRI* as well as 1 µmol/L *HpaII* or *MspI* adapter pairs, 0.5 U T4 DNA ligase with its corresponding buffer (Thermo Scientific), 0.05 mol/L NaCl and 0.5 µg BSA (BioLabs/NBA). The products were diluted 10 fold with 1:10 TE buffer (20 mmol/L Tris-HCl, pH 8.0; 0.1 mmol/L EDTA, pH 8.0) after an incubation for 2 h at 37 °C and a subsequent enzyme denaturation step at 70 °C for 15 min.

Preselective amplification was performed with 1 µl diluted DNA restriction-ligation product and 4 µl core mix. The core mix consisted of preselective *EcoRI* and *HpaII* or *MspI* primers (Biomers) with a single selective nucleotide and an AFLP core mix with 1× Buffer S, 0.2 mmol/L dNTPs, and 1.25 U Taq-Polymerase (PeqLab). PCR started at 94 °C for 2 min, followed by 30 cycles of 20 s denaturation at 94 °C, 30 s annealing at 56 °C and 2 min elongation at 72 °C. 2 min at 72 °C ended the elongation period and 30 min at 60 °C with a cool down to 4 °C completed the PCR run. The products were diluted 20 fold with 1:10 TE buffer.

A screening of 36 primer combinations with eight randomly selected individuals revealed three primer combinations for further analyses (Table S4.2). Selective amplification was performed in a total reaction volume of 5 µl, composed of 0.75 µl diluted preselective amplification product and 4.25 µl core mix, with 0.05 µmol/L selective *EcoRI* (Biomers) primers, 0.25 µmol/L *HpaII* or *MspI* (Biomers) primers and AFLP core mix containing 1× Buffer S, 0.2 mmol/L dNTPs, 1.25 U Taq-Polymerase (PeqLab). *EcoRI* primers were labelled with three different fluorescent dyes for fragment detection (Beckman dye D2, D3, and D4). Following PCR parameters were chosen: 2 min at 94 °C; 10 cycles of 20 s

denaturation at 94 °C, 30 s annealing at 66 °C (temperature was reduced every subsequent step by 1 °C), and 2 min elongation at 72 °C; then additional 25 cycles of 20 s denaturation at 94 °C, 30 s annealing at 56 °C, and 2 min elongation at 72 °C. The PCR was completed by a following 30 min step at 60 °C and a cool down to 4 °C. Selective PCR products were diluted with 1:10 TE buffer (Table S4.2).

Amplified selective PCR products of each individual (5 µl) were added to a stop solution, consisting of 2 µl sodium acetate (3 mol/L, pH 5.2), 2 µl Na_2EDTA (100 mmol/L, pH 8.0), and 1 µl glycogen (Roche). DNA was precipitated by adding 60 µl of ice-cold ethanol (96 %; -20 °C), immediate shaking and subsequent centrifugation at 4 °C for 20 min at 14,000 g. The pelleted DNA was washed once by adding 200 µl ice-cold ethanol (70 %; -20 °C) and centrifuged as described above. Afterwards, the DNA pellet was dried in a vacuum concentrator (Eppendorf). The pelleted DNA was redissolved in a mixture of 24.8 µl Sample Loading Solution (Beckman Coulter) and 0.2 µl CEQ Size Standard 400 (Beckman Coulter).

The fluorescence-labelled DNA fragments were separated by capillary gel electrophoresis using an automated capillary electrophoresis machine (GeXP, Beckmann Coulter). Results were examined with the GeXP software (Beckmann Coulter, USA). Data were exported into three curve-files, each representing one primer pair. These virtual gels were analysed manually using the software Bionumerics 7.6.2 (Applied Maths, Kortrijk, Belgium). Only strong and clearly defined fragments were taken into account for further analyses.

2 SUPPLEMENTARY MATERIAL - CHAPTER 2

Appendix A: Pearson correlation patterns (Table S2.7) showed a positive correlation between CON_1820 and AREA_1820. CON_2018 was also positively associated with AREA_1820, AREA_2018, and AREA_S. AREA_S and AREA_2018 were linked as well. Furthermore, DIST_1820 and DIST_2018 were positively correlated. Correlations between landscape variables originated from data collection. Since past landscape structures are the basis of their present counterparts, these variables generally showed intercorrelation patterns. Additionally, the total area of calcareous grasslands represented the sum of all single calcareous grassland areas within each 3 km circle (inclusively the area of the study site). These single areas formed the basis of the connectivity calculation. Therefore, the connectivity correlated with both AREA_S and the total area of calcareous grasslands. Although these variables showed intercorrelation patterns, each of these landscape variables was important to illustrate the impact of landscape on genetic diversity.

The cover of vascular plants and mosses significantly increased with DIST_1820, while the cover of open soil decreased with increasing DIST_1820. Thus, particularly DIST_1820 seemed to describe the movement patterns of livestock and the level of grazing intensity and disturbance in the present study. The intercorrelation patterns between the cover of vascular plants, mosses, litter, and open soil originated from data collection, setting the sum of these variables to 100 %.

The population size of *C. rotundifolia* was positively associated with the population size of *A. cynanchica* and AREA_S. Additionally, the population size of *L. catharticum* grew with increasing AREA_S as well as AREA_2018. All these intercorrelation patterns derived from the method of population size calculation.

Table S2.1: Number (No.), name (Population), geographic location (WGS84), and habitat age (Age) of all analysed populations.

No.	Population	La. (N)	Lo. (E)	Age
01	Bichishausen	48° 20' 06''	9° 30' 05''	ancient
02	Truchtelfingen	48° 14' 30''	9° 02' 41''	ancient
03	Mehrstetten	48° 23' 02''	9° 34' 08''	ancient
04	Merklingen	48° 30' 36''	9° 47' 21''	ancient
05	Burgfelden	48° 13' 46''	8° 56' 34''	ancient
06	Münsingen	48° 23' 44''	9° 30' 16''	ancient
07	Weidach	48° 26' 31''	9° 53' 09''	ancient
08	Lonsee	48° 32' 59''	9° 54' 55''	ancient
09	Unterdigisheim	48° 10' 01''	8° 54' 55''	ancient
10	Gomadingen	48° 23' 28''	9° 22' 37''	ancient
11	Aichen	48° 31' 21''	9° 47' 36''	recent
12	Meßstetten	48° 10' 26''	8° 57' 23''	recent
13	Wasserstetten	48° 22' 03''	9° 25' 55''	recent
14	Hausen ob Urspring	48° 24' 12''	9° 41' 26''	recent
15	Ehingen	48° 18' 29''	9° 43' 29''	recent
16	Ödenwaldstetten	48° 20' 27''	9° 23' 47''	recent
17	Oberstetten_1	48° 19' 13''	9° 18' 57''	recent
18	Oberstetten_2	48° 18' 40''	9° 20' 00''	recent
19	Ebingen	48° 13' 03''	8° 59' 16''	recent

Table S2.2: Map data used for habitat age determination and the analyses of past and present landscape structures.

Year	Name	Source	Accessed
1820-1850	Land Surveys of the Kingdom of Württemberg	Kohler, K. 1858. Die Landesvermessung des Königreichs Württemberg in wissenschaftlicher, technischer und geschichtlicher Beziehung. Cotta. (1:2,500)	20 July 2016
1875-1876	Land Surveys of the grand duchy of Baden	Landesarchiv Baden-Württemberg. Flurkarten des Königreichs Baden. http://www.landesarchiv-bw.de (1:10,000)	20 May 2018
1902-1914	Topographic Maps of the Kingdom of Württemberg	SLUB (Sächsische Landesbibliothek – Staats- und Universitätsbibliothek Dresden). 2018. Topographische Karten (Meßtischblätter) Deutschland 1870-1943. http://www.deutschefotothek.de/cms/kartenforum-sachsen-messtischblaetter.xml (1:25,000)	24 July 2016
1951-1953	Allied Nations Topographic Maps	Ritz, M. 2018. Landeskartenarchiv.de. https://www.landkartenarchiv.de/deutschland_topographischekarten.php (1:25,000)	30 July 2016
2017-2018	Current Topographic Maps	Landesamt für Geoinformation und Landentwicklung Baden-Württemberg (LGL). https://owsproxy.lgl-bw.de/owsproxy/ows/WMS_LGL-BW_ATKIS_DTK_25_K_A? (1:25,000)	11 April 2018

Table S2.3: Landscape structure of and around the analysed study sites.

No.	AREA_1820	DIST_1820	CON_1820	AREA_2018	DIST_2018	CON_2018	AREA_S
01	303.769	0.190	89.615	101.837	0.226	34.585	15.352
02	749.002	1.347	150.365	140.864	0.160	41.866	7.258
03	351.460	0.733	69.110	52.457	0.568	18.535	1.093
04	343.444	2.113	113.193	81.741	0.381	30.606	4.656
05	485.509	1.544	107.905	330.805	0.235	25.079	21.459
06	478.301	1.343	155.503	56.822	0.854	32.032	17.369
07	260.565	0.674	62.049	56.598	0.239	16.006	5.299
08	232.102	0.607	66.925	68.229	0.158	20.973	7.831
09	378.833	0.566	77.065	18.793	0.295	11.809	4.080
10	1119.053	1.094	264.069	77.629	0.265	23.943	5.692
11	280.422	0.915	52.888	70.109	0.192	27.454	3.363
12	330.566	0.673	41.370	268.342	0.424	44.308	17.704
13	488.244	0.273	85.271	104.469	0.237	25.695	13.675
14	123.200	1.469	31.258	16.243	0.482	6.679	2.696
15	196.789	0.924	41.566	15.631	0.614	7.750	2.020
16	137.422	1.800	16.995	15.464	0.419	10.688	8.069
17	212.857	1.517	28.127	12.568	0.466	3.974	0.944
18	207.371	2.061	42.177	12.568	1.256	9.742	8.810
19	479.150	0.511	72.910	67.280	0.294	27.625	13.924
Mean	**376.740**	**1.071**	**82.545**	**82.550**	**0.409**	**22.071**	**8.489**
SE	± 53.994	± 0.132	± 13.383	± 19.534	± 0.062	± 2.708	± 1.434

AREA_S, area size [ha]
AREA_1820/AREA_2018, past and present total area of calcareous grasslands [ha]
DIST_1820/DIST_2018, past and present distances to the nearest settlement [km]
CON_1820/CON_2018, past and present connectivity

Table S2.4: Habitat quality and calculated population size of *A. cynanchica*, *C. rotundifolia*, and *L. catharticum* per study site.

	Habitat quality				Population size		
No.	**VASC**	**MOSS**	**LITT**	**O_SOIL**	***A. cynanchica***	***C. rotundifolia***	***L. catharticum***
01	79.0	43.0	3.2	7.6	1954858.3	358220.1	10234.9
02	93.0	44.0	1.2	0.7	33871.7	24194.0	48388.1
03	75.4	47.0	2.2	7.6	34966.6	4370.8	22582.6
04	87.8	85.0	2.0	0.5	9311.4	6207.6	114841.2
05	87.0	77.0	3.0	1.0	42918.4	400571.4	314734.7
06	82.0	84.0	28.0	0.3	1505327.8	555813.3	46317.8
07	75.0	77.0	3.8	2.0	199726.2	48912.5	28532.3
08	64.0	44.0	37.0	3.0	182726.5	120077.4	36545.3
09	76.0	55.0	6.8	5.0	38075.4	5439.3	2719.7
10	88.0	88.0	9.4	0.5	64509.6	64509.6	34152.1
11	85.0	66.0	3.4	1.3	17936.5	2242.1	17936.5
12	83.6	56.0	3.2	1.2	7608.2	951.0	3804.1
13	64.0	64.0	52.0	0.7	107443.1	29302.7	68372.9
14	82.0	81.0	3.6	0.4	2695.9	64700.5	35046.1
15	79.0	57.0	10.0	7.4	106378.0	14812.1	1346.6
16	82.0	79.0	6.0	1.0	1597698.0	21517.8	75312.4
17	83.0	78.0	5.0	1.0	15097.3	943.6	943.6
18	86.0	80.6	7.0	0.7	581427.3	5873.0	23492.0
19	87.0	74.0	30.0	0.9	111393.6	18565.6	27848.4
Mean	**81.0**	**67.3**	**11.4**	**2.3**	**348103.7**	**91959.2**	**48060.6**
SE	± 1.7	± 3.6	± 3.3	± 0.6	± 140954.4	± 36865.2	± 16224.5

VASC, cover of vascular plants [%]; MOSS, cover of mosses [%]; LITT, cover of litter [%]; O_Soil, cover of open soil [%]

Table S2.5: Primer combinations for the selective amplification of *A. cynanchica*, *C. rotundifolia,* and *L. catharticum*. Further, the dilution of the selective amplification product is given.

Species	*MseI*	*EcoRI*	Dilution	Beckman dye
A. cynanchica	CTC	AAG	-	D3
	CAT	AGG	-	D3
	CTG	AGG	-	D3
C. rotundifolia	CAC	ACC	1 : 2	D2
	CAT	AGG	-	D3
	CTG	ACA	1 : 2	D4
L. catharticum	CTC	AAC	1 : 2	D2
	CTA	AGG	-	D3
	CAA	ACA	1 : 5	D4

Table S2.6: Significant ($p < 0.05$) differences between past and present landscape variables (Wilcoxon-Mann-Whitney tests).

Landscape structure	Mean	SE	p-value	
AREA_1820	376.74	235.36	< 0.001	***
AREA_2018	82.55	85.15		
DIST_1820	1.07	0.58	< 0.001	***
DIST_2018	0.41	0.27		
CON_1820	82.55	58.33	< 0.001	***
CON_2018	22.07	11.80		

Signif. code: $p \leq 0.001$ ***

AREA_1820/AREA_2018, past and present total area of calcareous grasslands [ha]
DIST_1820/DIST_2018, past and present distances to the nearest settlement [km]
CON_1820/CON_2018, past and present connectivity

Table S2.7: Significant ($p < 0.05$) intercorrelations (Pearson correlation coefficients) between the explanatory variables used in the linear models.

	Landscape structure							**Habitat quality**				**Population size**		
	AREA_1820	DIST_1820	CON_1820	AREA_2018	DIST_2018	CON_2018	AREA_S	VASC	MOSS	LITT	O_SOIL	Ac	Cr	Lc
Landscape structure														
AREA_1820	1													
DIST_1820		1												
CON_1820	+ 0.93		1											
AREA_2018				1										
DIST_2018		+ 0.49			1									
CON_2018	+ 0.47			+ 0.65		1								
AREA_S				+ 0.71		+ 0.59	1							
Habitat quality														
VASC		+ 0.58						1						
MOSS		+ 0.62							1					
LITT								- 0.61		1				
O_SOIL		- 0.50							- 0.67		1			
Population size														
Ac												1		
Cr							+ 0.65					+ 0.58	1	
Lc				+ 0.65			+ 0.49							1

AREA_S, area size [ha]; AREA_1820/AREA_2018, past and present total area of calcareous grasslands [ha]; DIST_1820/DIST_2018, past and present distances to the nearest settlement [km]; CON_1820/CON_2018, past and present connectivity; VASC, cover of vascular plants [%]; MOSS, cover of mosses [%]; LITT, cover of litter [%]; O_SOIL, cover of open soil [%]; Ac/Cr/Lc, population size of *A. cynanchica*, *C. rotundifolia*, and *L. catharticum*

3 SUPPLEMENTARY MATERIAL - CHAPTER 3

Appendix B: Pearson correlation patterns (Table S3.7) revealed a positive association of AREA_1800 with AREA_S and CON_1800. AREA_2018 correlated positively with CON_2018. The total area of litter meadows represented the sum of all litter meadow sites within each 3 km circle (inclusively AREA_S) forming the basis of connectivity calculation. Therefore, the total area of wet meadows correlated with both AREA_S and connectivity. DIST_2018 was linked to DIST_1800, since present landscape structures are based on their past counterparts. However, each of these landscape variables was important to illustrate the impact of landscape on genetic diversity despite these intercorrelations.

The cover of mosses significantly increased with AREA_1800 and CON_2018 indicating past and present distribution mechanisms. The negative intercorrelation between the cover of mosses and the cover of open soil originated from data collection setting the sum of all habitat quality variables to 100 %.

The population size of *S. pratensis* decreased with moss coverage, but increased with AREA_S. The population size of *A. sylvestris* was positively associated with the population size of *F. ulmaria*. All these intercorrelation patterns were based on the method of population size calculation.

Table S3.1: Number (No.), name (Population), and position (WGS84) of the analysed populations.

No.	Population	La. (N)	Lo. (E)
01	Arrisried	47° 45' 07''	9° 52' 06''
02	Schlier	47° 45' 09''	9° 39' 08''
03	Schwanden	47° 43' 12''	10° 2' 11''
04	Ratzenried	47° 43' 15''	9° 54' 14''
05	Liebenried	47° 45' 16''	9° 53' 15''
06	Argen	47° 40' 18''	10° 4' 17''
07	Kißlegg	47° 47' 19''	9° 52' 18''
08	Rotheidlen	47° 43' 20''	9° 42' 19''
09	Bremberg	47° 46' 21''	9° 54' 20''
10	Nitzenweiler	47° 36' 23''	9° 38' 22''
11	Wolfegg	47° 49' 25''	9° 46' 24''
12	Wangen im Allgäu	47° 40' 08''	9° 50' 07''
13	Hinteressach	47° 40' 10''	9° 41' 09''
14	Wolfegg	47° 49' 11''	9° 49' 10''
15	Rotenbach	47° 47' 13''	9° 50' 12''
16	Hüttenweiler	47° 36' 14''	9° 45' 13''
17	Vogt	47° 45' 17''	9° 47' 16''
18	Gwigg	47° 52' 22''	9° 43' 21''
19	Sigrazhofen	47° 46' 24''	9° 56' 23''
20	Edensbach	47° 45' 26''	9° 43' 25''

Table S3.2: Map data used for habitat age determination and the analyses of past and present landscape structures.

Year	Name	Source	Accessed
1823-1866	Land Surveys of the Kingdom of Württemberg	Kohler, K. 1858. Die Landesvermessung des Königreichs Württemberg in wissenschaftlicher, technischer und geschichtlicher Beziehung. Cotta. (1:2,500)	20 July 2016
1875-1876	Land Surveys of the grand duchy of Baden	Landesarchiv Baden-Württemberg. Flurkarten des Königreichs Baden. http://www.landesarchiv-bw.de (1:10,000)	20 May 2018
1808-1864	Historical cadastral maps of Bavaria	https://geoportal.bayern.de/bayernatlas	20 May 2018
1857	Historical cadastral maps of Vorarlberg (Austria)	http://vogis.cnv.at/atlas3/init.aspx?karte=basiskarten_und_bilder	20 May 2018
1910-1920	Topographic Maps of the Kingdom of Württemberg	SLUB (Sächsische Landesbibliothek – Staats- und Universitätsbibliothek Dresden). 2018. Topographische Karten (Meßtischblätter) Deutschland 1870-1943. http://www.deutschefotothek.de/cms/kartenforum-sachsen-messtischblaetter.xml (1:25,000)	24 July 2016
1951-1953	Allied Nations Topographic Maps	Ritz, M. 2018. Landeskartenarchiv.de. https://www.landkartenarchiv.de/deutschland_topographischekarten.php (1:25,000)	30 July 2016
2017-2018	Current Topographic Maps	Landesamt für Geoinformation und Landentwicklung Baden-Württemberg (LGL). https://owsproxy.lgl-bw.de/owsproxy/ows/WMS_LGL-BW_ATKIS_DTK_25_K_A? (1:25,000)	11 April 2018

Table S3.3: Past and present landscape structure of and around the analysed study sites.

No.	AREA_1800	DIST_1800	CON_1800	AREA_2018	DIST_2018	CON_2018	AREA_S
01	144.063	0.415	161.712	15.219	0.255	7.706	3.769
02	55.109	0.816	22.880	25.040	0.562	14.042	7.345
03	101.816	0.321	19.601	183.627	0.286	27.944	1.487
04	45.189	0.239	20.868	19.146	0.449	3.625	0.354
05	146.429	0.229	31.732	24.568	0.231	6.621	2.204
06	141.307	0.344	45.371	30.241	0.470	18.393	3.590
07	97.989	0.264	29.213	21.987	0.298	7.324	2.530
08	39.178	0.364	10.741	32.398	0.223	11.161	1.091
09	103.673	0.336	47.789	41.218	0.276	9.369	2.396
10	108.528	0.328	32.125	75.885	0.308	21.152	2.470
11	69.767	0.347	10.567	6.463	0.346	2.112	1.817
12	109.420	0.322	28.842	33.390	0.303	12.750	3.696
13	94.742	0.525	13.116	49.780	0.498	13.882	0.637
14	60.067	0.498	7.130	12.674	0.507	4.738	3.658
15	111.016	0.409	19.837	26.164	0.396	11.030	7.237
16	203.294	0.127	29.672	45.781	0.132	17.924	7.562
17	29.230	0.322	3.123	37.518	0.277	9.394	1.748
18	114.972	1.178	38.507	28.965	0.682	17.332	3.308
19	95.027	0.319	36.660	37.024	0.298	15.482	0.908
20	55.344	0.311	20.797	54.622	0.309	13.524	1.315
Mean	**96.308**	**0.401**	**31.514**	**40.086**	**0.355**	**12.275**	**2.956**
SE	± 9.60	± 0.05	± 7.38	± 8.35	± 0.03	± 1.42	± 0.49

AREA_S, area size [ha]
AREA_1800/AREA_2018, past and present total area of wet meadows [ha]
DIST_1800/DIST_2018, past and present distances to the nearest settlement [km]
CON_1800/CON_2018, past and present connectivity

Table S3.4: Habitat quality of the analysed study sites as well as population size per species and investigated population.

	Habitat quality				Population size		
No.	**VASC**	**MOSS**	**LITT**	**O_SOIL**	***A. sylvestris***	***F. ulmaria***	***S. pratensis***
01	73.0	69.0	9.6	1.0	50,252.1	2,512.6	40,201.7
02	87.0	36.0	23.0	2.2	4,896.8	142,006.9	53,864.7
03	77.0	78.0	2.6	0.6	0.0	4,957.8	991.6
04	86.0	34.0	9.8	0.4	354.1	18,415.5	10,978.4
05	84.0	67.0	12.6	0.0	5,878.0	74,944.9	1,469.5
06	76.0	59.0	10.0	2.2	150,776.7	222,575.2	59,832.0
07	71.0	56.0	7.2	1.8	31,625.2	168,667.5	2,108.3
08	79.5	62.0	11.1	2.6	11,635.1	37,087.0	13,089.5
09	87.0	55.5	3.0	3.1	4,791.0	62,283.3	7,985.0
10	80.0	71.0	1.8	1.6	67,503.2	306,234.2	13,171.4
11	81.0	63.0	2.2	3.8	23,618.9	350,650.1	0.0
12	84.0	72.0	18.4	0.0	61,595.6	359,718.5	24,638.3
13	87.0	78.0	3.4	0.6	14,439.0	33,549.4	1,698.7
14	75.0	16.0	30.0	6.6	33,529.3	198,127.6	85,347.3
15	94.0	58.0	10.6	2.0	50,661.5	260,544.9	36,186.8
16	80.0	61.0	7.2	3.2	20,164.3	151,232.1	25,205.3
17	81.0	36.0	12.0	7.8	75,751.6	48,947.2	3,496.2
18	76.0	66.0	7.8	1.0	11,027.6	114,686.9	2,205.5
19	67.5	63.3	4.5	5.7	5,296.6	27,239.8	0.0
20	83.0	72.0	58.0	0.0	876.4	81,503.5	0.0
Mean	**80.5**	**58.6**	**12.2**	**2.3**	**31233.7**	**133294.2**	**19123.5**
SE	± 1.4	± 3.6	± 2.9	± 0.5	± 8282.6	± 25866.5	± 5423.1

VASC, cover of vascular plants [%]; MOSS, cover of mosses [%]; LITT, cover of litter [%]; O_SOIL, cover of open soil [%]

Table S3.5: Primer combinations and respective dilutions of the selective amplification of *A. sylvestris*, *F. ulmaria*, and *S. pratensis*.

Species	*MSeI*	*EcoRI*	Dilution	Beckman dye
A. sylvestris	CTC	ACC	1:2	D2
	CAC	ACG	-	D3
	CTC	ACA	1:5	D4
F. ulmaria	CAA	AAC	1:2	D2
	CAA	AAG	-	D3
	CAT	ACT	1:5	D4
S. pratensis	CAC	ACC	1:2	D2
	CTC	ACG	-	D3
	CTC	ACT	1:5	D4

Table S3.6: Significant ($p < 0.05$) differences between past (1800) and present (2018) landscape variables (Wilcoxon-Mann-Whitney tests).

Landscape structure	Mean	SE	p-value	
AREA_1800	96.31	42.93	< 0.001	***
AREA_2018	40.09	37.34		
DIST_1800	0.40	0.23	0.383	n.s.
DIST_2018	0.36	0.13		
CON_1800	31.51	33.00	< 0.001	***
CON_2018	12.28	6.36		

Signif. codes: $p \leq 0.001$ ***; $p > 0.05$ n.s.

AREA_1800/AREA_2018, past and present total area of wet meadows [ha]
DIST_1800/DIST_2018, past and present distance to the nearest settlement [km]
CON_1800/CON_2018, past and present connectivity

Table S3.7: Significant (p < 0.05) correlations (Pearson correlation coefficients) between the explanatory variables used in the linear models.

	Landscape structure							**Habitat quality**				**Population size**		
	AREA_1800	DIST_1800	CON_1800	AREA_2018	DIST_2018	CON_2018	AREA_S	VASC	MOSS	LITT	O_SOIL	As	Fu	Sp
Landscape structure														
AREA_1800	1													
DIST_1800		1												
CON_1800	0.47		1											
AREA_2018				1										
DIST_2018		0.82			1									
CON_2018				0.78		1								
AREA_S	0.45						1							
Habitat quality														
VASC								1						
MOSS	0.46					0.50			1					
LITT										1				
O_SOIL									- 0.60		1			
Population size														
As												1		
Fu												0.46	1	
Sp							0.53		- 0.53					1

AREA_S, area size [ha]; AREA_1800/AREA_2018, past and present total area of wet meadows [ha]; DIST_1800/DIST_2018, past and present distance to the nearest settlement [km]; CON_1800/CON_2018, past and present connectivity; VASC, cover of vascular plants [%]; MOSS, cover of mosses [%]; LITT, cover of litter [%]; O_Soil, cover of open soil [%]; As/Fu/Sp, population size of *A. sylvestris*, *F. ulmaria*, and *S. pratense*

4 SUPPLEMENTARY MATERIAL - CHAPTER 4

Table S4.1: Number (No.), name (Population), and geographic location (WGS84) of all analysed populations. Also specified is the number of investigated individuals (N). Further, the habitat type, the natural region, and the number of the adjacent population in the paired plot design are given.

No.	Population	La. (N)	Lo. (E)	N	Habitat type	Natural region	Adjacent population
01	Truchtelfingen	48° 14' 30''	9° 02' 41''	16	calcareous grassland	Hohe Schwabenalb	08
02	Oberwilzingen	48° 14' 31''	9° 30' 42''	16	calcareous grassland	Mittlere Flächenalb	09
03	Münsingen	48° 23' 44''	9° 30' 16''	16	calcareous grassland	Mittlere Kuppenalb	10
04	Weidach	48° 26' 31''	9° 53' 09''	16	calcareous grassland	Mittlere Flächenalb	06
05	Gomadingen	48° 23' 28''	9° 22' 37''	16	calcareous grassland	Mittlere Kuppenalb	07
06	Blaubeuren	48° 25' 29''	9° 45' 38''	16	oat-grass meadow	Mittlere Flächenalb	04
07	Greuthau	48° 23' 30''	9° 15' 39''	16	oat-grass meadow	Mittlere Kuppenalb	06
08	Neufra	48° 15' 31''	9° 10' 40''	16	oat-grass meadow	Mittlere Kuppenalb	01
09	Rechtenstein	48° 14' 32''	9° 33' 41''	16	oat-grass meadow	Mittlere Flächenalb	02
10	Münsingen	48° 23' 33''	9° 34' 42''	16	oat-grass meadow	Mittlere Flächenalb	03

Table S4.2: Adaptor- and primer sequences used for AFLP and MSAP analyses.

Primer	Sequence (5' - 3')	Dilution
Adaptors		
EcoRI-adapter top	CTC GTA GAC TGC GTA CC	
EcoRI-adapter bottom	AAT TGG TAC GCA GTC TAC	
MSeI-adaptor top (AFLP)	GAC GAT GAG TCC TGA G	
MSeI-adaptor bottom (AFLP)	TAC TCA GGA CTC AT	
HpaII/MspI-adaptor top (MSAP)	GAT CAT GAG TCC TGC T	
HpaII/MspI-adaptor bottom (MSAP)	CGA GCA GGA CTC ATG A	
Preselective primers		
EcoRI + A	GAC TGC GTA CCA ATT CA	
MSeI + C (AFLP)	GAT GAG TCC TGA GTA AC	
HpaII/MspI (MSAP)	ATC ATG AGT CCT GCT CGG	
Selective primer AFLP		
EcoRI + AAC [1]	(dyeD2) GAC TGC GTA CCA ATT CAA C	1 : 2
EcoRI + AAG [2]	(dyeD3) GAC TGC GTA CCA ATT CAA G	-
EcoRI + ACA [3]	(dyeD4) GAC TGC GTA CCA ATT CAC A	1 : 5
MSeI + CAA [1, 2]	GAT GAG TCC TGA GTA ACA A	
MSeI + CAC [3]	GAT GAG TCC TGA GTA ACA C	
Selective primer MSAP		
EcoRI + AAC [1]	(dyeD2) GAC TGC GTA CCA ATT CAA C	1 : 2
EcoRI + AAG [2]	(dyeD3) GAC TGC GTA CCA ATT CAA G	-
EcoRI + ACA [3]	(dyeD4) GAC TGC GTA CCA ATT CAC A	1 : 5
HpaII/MspI + AAT [1]	ATC ATG AGT CCT GCT CGG AAT	
HpaII/MspI + TCC [2,3]	ATC ATG AGT CCT GCT CGG TCC	

Superscript numbers indicate primer combinations used for the selective amplification

Table S4.3: Pairwise population distance matrices (Φ_{PT}) for (a) 124 AFLP loci (upper diagonal) and 408 MSAP loci (lower diagonal), (b) 116 MSAP h-epiloci (upper diagonal) and 144 MSAP m-epiloci (lower diagonal), and (c) 148 MSAP u-epiloci.

(a)

	01	**02**	**03**	**04**	**05**	**06**	**07**	**08**	**09**	**10**
01	-	0.012	0.053	0.067	0.026	0.126	0.068	0.057	0.068	0.082
02	0.037	-	0.019	0.062	0.039	0.103	0.046	0.071	0.067	0.083
03	0.061	0.048	-	0.055	0.010	0.110	0.045	0.104	0.074	0.083
04	0.059	0.031	0.050	-	0.046	0.064	0.040	0.052	0.049	0.042
05	0.069	0.040	0.022	0.032	-	0.106	0.051	0.051	0.059	0.051
06	0.060	0.049	0.047	0.021	0.032	-	0.059	0.050	0.034	0.035
07	0.088	0.065	0.054	0.056	0.029	0.049	-	0.039	0.063	0.045
08	0.061	0.048	0.051	0.033	0.027	0.024	0.042	-	0.009	0.024
09	0.068	0.052	0.053	0.048	0.035	0.047	0.050	0.017	-	0.044
10	0.068	0.062	0.040	0.047	0.040	0.028	0.062	0.027	0.022	-

(b)

	01	**02**	**03**	**04**	**05**	**06**	**07**	**08**	**09**	**10**
01	-	0.078	0.100	0.099	0.091	0.104	0.097	0.150	0.132	0.161
02	0.030	-	0.038	0.075	0.039	0.092	0.063	0.100	0.098	0.122
03	0.051	0.043	-	0.074	0.000	0.088	0.017	0.081	0.078	0.106
04	0.045	0.008	0.042	-	0.022	0.018	0.043	0.060	0.086	0.068
05	0.058	0.032	0.027	0.015	-	0.032	0.003	0.059	0.050	0.082
06	0.046	0.022	0.030	0.014	0.022	-	0.038	0.085	0.088	0.069
07	0.087	0.067	0.061	0.047	0.038	0.046	-	0.027	0.032	0.056
08	0.037	0.035	0.043	0.019	0.015	0.014	0.045	-	0.043	0.041
09	0.053	0.031	0.047	0.028	0.023	0.027	0.053	0.007	-	0.026
10	0.048	0.042	0.029	0.041	0.033	0.021	0.062	0.019	0.020	-

(c)

	01	02	03	04	05	06	07	08	09	10
01	-									
02	0.030	-								
03	0.058	0.056	-							
04	0.060	0.040	0.051	-						
05	0.071	0.048	0.024	0.050	-					
06	0.059	0.060	0.050	0.028	0.043	-				
07	0.086	0.063	0.058	0.069	0.029	0.056	-			
08	0.059	0.047	0.051	0.038	0.029	0.017	0.044	-		
09	0.060	0.057	0.051	0.055	0.042	0.052	0.054	0.019	-	
10	0.056	0.064	0.031	0.046	0.035	0.022	0.063	0.031	0.022	-

Table S4.4: Pairwise habitat dissimilarity (upper diagonal) and geographic distance (km) matrix (lower diagonal).

	01	02	03	04	05	06	07	08	09	10
01	-	0	0	0	0	1	1	1	1	1
02	9.83	-	0	0	0	1	1	1	1	1
03	6.75	16.56	-	0	0	1	1	1	1	1
04	30.82	35.15	28.64	-	0	1	1	1	1	1
05	11.11	18.92	9.43	38.01	-	1	1	1	1	1
06	20.90	26.27	18.57	10.10	27.97	-	0	0	0	0
07	19.05	24.86	18.10	46.56	8.68	36.57	-	0	0	0
08	25.78	25.34	28.83	56.55	20.98	46.54	16.15	-	0	0
09	11.45	3.50	17.85	33.13	21.57	24.79	28.02	28.78	-	0
10	8.36	16.96	4.89	23.88	14.31	13.78	22.99	33.05	17.28	-

Table S4.5: Number of loci and methylation pattern per selective primer combination.

EcoRI selective primer	MspI/HpaII selective primer	h-epiloci	m-epiloci	u-epiloci	Loci per primer combination
AAC	AAT	39	48	49	136
AAG	TCC	35	46	47	128
ACA	TCC	42	50	52	144
Total		**116 (28.4 %)**	**144 (35.3 %)**	**148 (36.3 %)**	**408**

Table S4.6: Results of simple and partial Mantel tests for genetic and epigenetic pairwise population Φ_{PT} with geographic distance (km) and habitat dissimilarity matrices partialled on genetic and epigenetic distance matrices.

	AFLP		MSAP	
	r	**p**	**r**	**p**
Geographic distance matrix				
Simple test	- 0.08	0.652	- 0.16	0.795
Partialled on AFLP/MSAP	- 0.04	0.565	- 0.14	0.764
Habitat dissimilarity distance matrix				
Simple test	0.51	0.004	0.20	0.113
Partialled on AFLP/MSAP	0.48	0.005	0.07	0.350

p values were calculated with 9,999 permutations

Table S4.7: Pearson correlation matrix with correlation coefficients (upper diagonal) and p-values (lower diagonal).

		SI_MSAP				EIV			
	SI_AFLP	all epiloci	h-epiloci	m-epiloci	u-epiloci	L	M	R	N
SI_AFLP	-	0.07	0.10	- 0.03	0.11	0.03	- 0.10	0.00	- 0.21
SI_MSAP									
all subepiloci	0.85	-	0.82	0.94	0.95	- 0.36	0.66	- 0.71	0.49
h-subepiloci	0.79	0.00	-	0.62	0.61	0.21	0.24	- 0.42	0.07
m-subepiloci	0.93	0.00	0.06	-	0.93	- 0.57	0.74	- 0.72	0.62
u-subepiloci	0.77	0.00	0.06	0.00	-	- 0.57	0.78	- 0.78	0.62
EIV									
L	0.93	0.31	0.57	0.09	0.09	-	- 0.82	0.70	- 0.82
M	0.77	0.04	0.51	0.01	0.01	0.00	-	- 0.89	0.96
R	0.99	0.02	0.23	0.02	0.01	0.02	0.00	-	- 0.80
N	0.56	0.15	0.85	0.05	0.05	0.00	0.00	0.01	-

SI, Shannon information index; EIV, Ellenberg indicator value; L, light; M, soil moisture; R, soil reaction/pH; N, soil nitrogen